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Research Article

The humoral hypercalcemia of malignancy (HHM) is caused by tumor cells that release a circulating factor which stimulates osteoclastic bone resorption. Recently, it has been reported that tumors associated with HHM contain factors that stimulate renal and bone cell adenylate cyclase. The activity was inhibited by parathyroid hormone (PTH) antagonists, and this led to the hypothesis that hypercalcemia is due to bone resorbing factors that engage PTH receptors in bone. Since it is not known whether the bone resorbing factors act via PTH receptors in bone, we examined the effects of PTH antagonists on PTH-stimulated bone resorption and bone resorbing activity that was produced by two tumor models of HHM which also release these adenylate cyclase stimulating factors. The PTH antagonists [8,18norleucine, 34tyrosine]bovine PTH (3-34) amide and [34tyrosine]bovine PTH (7-34) completely inhibited PTH-stimulated bone resorption. Neither antagonist inhibited bone resorption that was stimulated by the conditioned medium from cells that were derived from the Walker rat 256 tumor model of HHM. Both antagonists also failed to inhibit bone resorption that was stimulated by culture media from cells that were derived from the rat Leydig cell tumor. These data suggest that in these two models of HHM, the bone resorbing factors do not exert their effects by interacting with PTH receptors on bone.

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Failure of Parathyroid Hormone Antagonists to Inhibit In Vitro Bone Resorbing Activity Produced by Two Animal Models of the Humoral Hypercalcemia of Malignancy

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Abstract. The humoral hypercalcemia of malignancy (HHM) is caused by tumor cells that release a circulating factor which stimulates osteoclastic bone resorption. Recently, it has been reported that tumors associated with HHM contain factors that stimulate renal and bone cell adenylate cyclase. The activity was inhibited by parathyroid hormone (PTH) antagonists, and this led to the hypothesis that hypercalcemia is due to bone resorbing factors that engage PTH receptors in bone. Since it is not known whether the bone resorbing factors act via PTH receptors in bone, we examined the effects of PTH antagonists on PTH-stimulated bone resorption and bone resorbing activity that was produced by two tumor models of HHM which also release these adenylate cyclase stimulating factors. The PTH antagonists [^{8,18}norleucine, ³⁴tyrosine]bovine PTH (3-34) amide and [³⁴tyrosine]bovine PTH (7-34) completely inhibited PTH-stimulated bone resorption. Neither antagonist inhibited bone resorption that was stimulated by the conditioned medium from cells that were derived from the Walker rat 256 tumor model of HHM. Both antagonists also failed to inhibit bone resorption that was stimulated by culture media from cells that were derived from the rat Leydig cell tumor. These data suggest that

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Introduction

The hypothesis that humoral hypercalcemia of malignancy (HHM)¹ (1) is due to parathyroid hormone (PTH)-like factors which cause bone resorption by binding to PTH receptors is based on the observation that tumors associated with hypercalcemia produce factors which stimulate cyclase activity and which are inhibited by PTH antagonists (2-4). If this hypothesis is correct, then these PTH antagonists should inhibit the bone-resorbing factors produced by these tumors, which are the primary cause of the hypercalcemia. To test the hypothesis, we examined two tumor models of HHM (5, 6) which produced both bone-resorbing activity and factors which stimulate adenylate cyclase to determine if the bone-resorbing activity was mediated via PTH receptors.

Methods

Tumor cell culture. Tumor cells were derived from explant cultures of the Walker 256 tumor. Rats that bore the tumors had serum calciums in the range of 12-17 mg/dl. Cells were plated in modified Eagle's medium (MEM) that contained 10% (vol/vol) heat-inactivated fetal calf serum (FCS) penicillin (100 U/ml), streptomycin (100 µg/ml), and were incubated at 37°C in a humidified 5% CO₂/95% air atmosphere. Subconfluent cells were transferred into MEM that contained 2% (vol/vol) FCS, and 48 h later the tumor cell conditioned medium was

1. Abbreviations used in this paper: [³⁴Tyr]bPTH (7-34) amide, [³⁴Tyrosine]bovine PTH (7-34) amide; EGF, epidermal growth factor; FCS, fetal calf serum; HHM, humoral hypercalcemia of malignancy; MEM, modified Eagle's medium; [^{8,18}Nle, ³⁴Tyr] bPTH (3-34) amide, [^{8,18}norleucine, ³⁴tyrosine] bovine PTH (3-34) amide; PTH, parathyroid hormone.

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collected, centrifuged at 1,000 g for 15 min, and the supernatant was stored at -20°C before assay.

Cells that were derived from explant cultures of the Leydig cell tumor were cultured in RPMI 1640 medium that contained 5% (vol/vol) FCS and 5% (vol/vol) horse serum. Rats that bore tumors developed serum calciums between 13 and 18 mg/dl (6). For collection of conditioned medium, subconfluent cells were cultured in RPMI that contained 1% FCS and 1% horse serum for 48 h and then were treated as described for the Walker cells.

Assay for bone resorption. The assay for bone resorption that was used in these experiments was as previously described (7). Rats at the 18th d of gestation were injected subcutaneously with 200 μ Ci $^{45}\text{CaCl}_2$. The following day the fetuses were removed and the tubular mineralized shafts of the radii and ulnae were dissected free from the cartilaginous ends and surrounding soft tissue. The explanted shafts were cultured in BGJ(b) medium to allow for exchange of loosely complexed ^{45}Ca with stable calcium in the medium. The bones were then cultured for 48 h in BGJb medium that was supplemented with 5% FCS (heat inactivated) in the absence or presence of test substances. In all experiments, the controls consisted of MEM that contained 2% FCS or RPMI 1640 that contained 1% FCS and 1% horse serum, and were treated identically to the tumor cell conditioned medium. The extent of bone resorption was measured by the release of total radioactivity from individual bones into the medium during the culture period. Four bones were used in each treatment group and statistical differences between the means were determined using the *t* test for unpaired samples.

PTH and PTH antagonists. Bovine PTH (1-84) was obtained from the National Institutes of Health, Bethesda, MD. [$^{8,18}\text{Nle}$, ^{34}Tyr]bPTH (3-34) amide and [^{34}Tyr]bPTH (7-34) amide were purchased from Bachem Fine chemicals (Torrance, CA) and were added simultaneously with test or control samples at the final concentrations that are indicated in each table or figure legend.

Results

Table I shows the effects of [$^{8,18}\text{Nle}$, ^{34}Tyr]bPTH (3-34) amide at concentrations that ranged from 10^{-8} M to 10^{-6} M on bone resorption that was stimulated by Walker tumor cell conditioned medium. bPTH (1-84) at 4×10^{-8} M was used as a positive control. The antagonist (10^{-6} M) inhibited PTH-mediated bone resorption when added to the bones at a molar ratio of 25:1 with PTH (Table I). The antagonist had no inhibitory effect on the bone resorption that was stimulated by conditioned medium.

In our hands, the antagonist did not demonstrate any PTH-like agonist or partial agonist activity. However, since the (3-34) antagonist has been shown to have no antagonist activity when tested against PTH in vivo (8, 9), we repeated the experiments using the more recently developed [^{34}Tyr]bPTH (7-34) amide, which has been shown to inhibit PTH action in vivo (8). However, [^{34}Tyr]bPTH (7-34) amide at concentrations between 10^{-8} M and 10^{-6} M had no effect on the bone resorption that was stimulated by Walker rat tumor conditioned medium (Table II). This antagonist also inhibited PTH-stimulated bone resorption (Table III).

Fig. 1 shows the effects of bPTH (7-34) amide on resorption

Table I. The Effect of [$^{8,18}\text{Nle}$, ^{34}Tyr]bPTH (3-34) Amide on Bone Resorption Stimulated by Walker Conditioned Medium

Antagonist	% ^{45}Ca release				4×10^{-8} M
	Concentration	TCM	Control medium	PTH	
0	26 \pm 2*	15 \pm 1		34 \pm 6*	17 \pm 1
10^{-6} M	25 \pm 2*	14 \pm 1		17 \pm 1	19 \pm 1
10^{-7} M	22 \pm 2*	15 \pm 3		NT	NT
10^{-8} M	21 \pm 1*	11 \pm 1		NT	NT

Tumor cell conditioned medium (TCM) and control medium (MEM plus 2% FCS) (TCM) were diluted one in two with BGJ plus 5% FCS. Conditioned or control medium or PTH and antagonist were added together. PTH was added to BGJ plus 5% FCS to give a final concentration of 4×10^{-8} M. Values shown are mean \pm SEM ($n = 4$ bones). NT, not tested.

* Significantly greater than corresponding control ($P < 0.001$).

that was stimulated by Leydig tumor cell conditioned medium. Again, the antagonist had no effect on the bone resorption that was stimulated by the conditioned medium. bPTH 1-84 (8×10^{-8} M) was used as a positive control. In a separate experiment (data not shown), we also found that the (3-34) antagonist had no inhibitory activity on the bone-resorbing activity in the Leydig tumor cell conditioned medium.

Table III shows the effects of bPTH (7-34) on PTH (1-84)-stimulated bone resorption. The antagonist inhibited PTH-stimulated bone resorption when added to bones at a molar ratio of 25:1 with PTH. When added at a molar ratio of 2.5:1, there was only partial inhibition and the resorbing activity was ~70% of that obtained with PTH (1-84) alone. Antagonist at 10^{-8} M had no inhibitory effect on PTH-stimulated resorption.

Table II. The Effect of [^{34}Tyr]bPTH (7-34) Amide on Bone Resorption Stimulated by Walker Conditioned Medium

Antagonist	% ^{45}Ca release				
	Concentration	Experiment 1		Experiment 2	
		TCM	Control	TCM	Control
0	19 \pm 1*	9 \pm 1		19 \pm 4*	10 \pm 1
10^{-6} M	17 \pm 1*	9 \pm 1		16 \pm 1*	10 \pm 1
10^{-7} M	18 \pm 1*	9 \pm 1		15 \pm 1*	8 \pm 1
10^{-8} M	17 \pm 1*	8 \pm 1		17 \pm 2*	7 \pm 1

Tumor cell conditioned medium (TCM) and control medium were diluted one in two with BGJ plus 5% FCS. Conditioned medium and antagonist were added together and bones were incubated for 48 h. Values shown are mean \pm SEM ($n = 4$ bones).

* Significantly greater than control medium ($P < 0.005$).

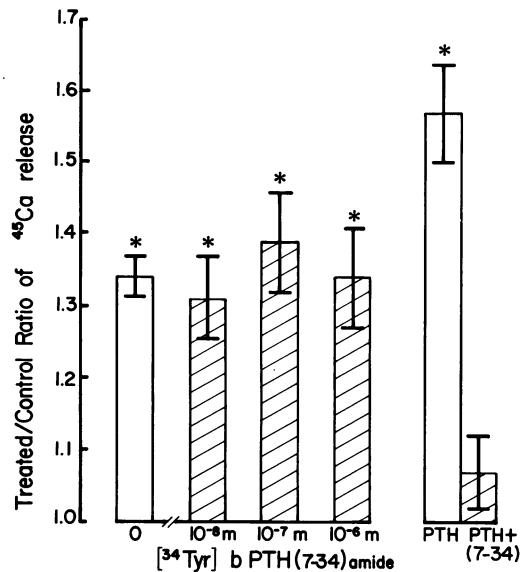


Figure 1. The effects of $[^{34}\text{Tyr}]b\text{PTH}(7-34)$ amide on bone resorption stimulated by Leydig tumor cell conditioned medium (TCM). TCM and control medium (RPMI plus 2% FCS) were diluted one in two with BGJ plus 5% FCS. Conditioned or control medium and antagonist were added together and bones were incubated for 48 h. Values shown represent pooled results from two separate experiments ($n = 8$ bones) and are expressed as treated: control ratios of ^{45}Ca released by TCM alone (□) or TCM plus antagonist (▨). PTH ($8 \times 10^{-8}\text{ M}$) in the absence (□) or presence (▨) of antagonist ($2 \times 10^{-6}\text{ M}$) was used as a positive control. * Significantly greater than corresponding control ($P < 0.005$).

Discussion

The nature of the humoral mediators of bone resorption in HHM have been the subject of much research. Since many

Table III. The Effect of $[^{34}\text{Tyr}]b\text{PTH}(7-34)$ Amide on $b\text{PTH}(1-84)$ -Mediated Bone Resorption

Antagonist	% ^{45}Ca release			
	Experiment 1		Experiment 2	
Concentration	PTH	Control	PTH	Control
0	20±2.0*	12±1	35±2	11±1
10^{-6} M	10±1‡	11±1	15±1‡	10±1
10^{-7} M	14±1‡	11±1	25±1‡	12±1
10^{-8} M	22±3*	11±2	37±3	12±1

PTH was used at $4 \times 10^{-8}\text{ M}$. Control medium consisted of BGJ plus 5% FCS. Control medium or PTH and antagonist were added together. Values shown are mean±SEM ($n = 4$ bones).

* Significantly greater than corresponding control ($P < 0.025$).

‡ Significantly less than PTH alone.

features of this syndrome (e.g., hypercalcemia, increased nephrogenous cyclic AMP, renal phosphate wasting) are also seen in primary hyperparathyroidism, the idea that hypercalcemia in many of these patients may be due to a PTH-like substance has been attractive.

Recently there have been several reports which showed that tumors associated with HHM contain a factor(s) which activates adenylate cyclase by interacting with PTH receptors (2-4). In collaborative studies, Rodan et al. (4) showed that Walker rat 256 and rat Leydig tumor cells both produce factors that stimulate adenylate cyclase activity in a rat osteosarcoma cell line, and this correlated positively with bone-resorbing activity that was produced by the same tumor cells. The cyclase stimulating activity that was produced by the Walker cells could be partially inhibited, and that produced by the Leydig cells was totally inhibited by the competitive PTH antagonist $[^{8,18}\text{Nle}, ^{34}\text{Tyr}]b\text{PTH}(3-34)$ amide (10^{-6} M). These data suggest that the cyclase stimulating factors were interacting with PTH receptors on the cells.

Since hypercalcemia of malignancy is due to increased osteoclastic bone resorption, it was important to determine whether the bone-resorbing factors that are produced by tumor models of HHM are also acting via PTH receptors and could be inhibited by PTH antagonists. We therefore examined the effects of two PTH antagonists, $[^{8,18}\text{Nle}, ^{34}\text{Tyr}]b\text{PTH}(3-34)$ amide and $[^{34}\text{Tyr}]b\text{PTH}(7-34)$ amide, on bone resorption stimulated by $b\text{PTH}(1-84)$ and by the resorptive factors produced by rat tumors associated with HHM. The (3-34) antagonist possesses an avidity for PTH receptors comparable to that of PTH (8) and has been shown to inhibit PTH-stimulated adenylate cyclase in canine renal cortical membranes (9). However, it has partial agonist activity in some systems and was found to be devoid of antagonist activity in vivo (8, 9). The (7-34) antagonist has been shown to inhibit PTH-stimulated excretion of urinary phosphate and cyclic AMP in vivo. We have shown here that both the (3-34) and (7-34) antagonists inhibited $b\text{PTH}(1-84)$ -mediated bone resorption (Fig. 1, Tables I and III).

Our data clearly show that bone-resorbing activity produced by the two tumor cell lines is not inhibited by the PTH antagonists. These data indicate that the bone-resorbing factors that were produced by the Walker and Leydig D6 tumor cells do not exert their biological effects on bone by interacting with PTH receptors on bone. Lack of inhibition of resorption cannot be due to use of supramaximal doses of conditioned medium, since a one in four dilution of medium results in the loss of 50% of activity in the Walker medium and almost total loss of activity in the Leydig medium. Furthermore, the antagonists (10^{-6} M - 10^{-8} M) inhibited cyclase activity in the same conditioned media tested at the same dilutions (one in two) (Gutierrez, G., and M. Katz, unpublished data). To exclude the possibility that failure to inhibit resorption was due to breakdown of the (7-34) antagonist in conditioned medium during the culture period, we incubated control and

tumor conditioned medium (diluted one in two with BGJ plus 5% FCS) alone or with (7-34) antagonist at 2×10^{-5} M for 48 h. These samples were then diluted 10-fold and tested for their ability to inhibit PTH (8×10^{-8} M)-mediated bone resorption. The antagonist (final concentration 2×10^{-6} M) preincubated for 48 h with conditioned medium was as effective in blocking PTH-mediated bone resorption as was fresh antagonist added to bones. This indicates that the antagonist is not altered or degraded in the culture medium in a way that affects its antagonist effects on PTH-mediated bone resorption.

It should be noted that PTH itself does not necessarily stimulate bone resorption by a cyclic nucleotide-mediated mechanism. It has recently been shown that the cyclic AMP and resorptive responses to bPTH and bPTH fragments in fetal rat calvaria can be dissociated, since some biologically active PTH fragments that stimulate bone resorption do not stimulate adenylate cyclase activity in the calvaria (10).

We have presented data (11) that in the case of the Leydig D6 tumor, a different type of mechanism is responsible for hypercalcemia. In this case, partially purified fractions of the bone-resorbing activity compete with ^{125}I epidermal growth factor (EGF) for binding to EGF receptors. Our hypothesis is that in this tumor model, the bone-resorbing factor that is responsible for hypercalcemia depends on occupancy of the EGF receptor for its effects.

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