Similarity of Synthetic Peptide from Human Tumor to Parathyroid Hormone in Vivo and in Vitro

NOBORU HORIUCHI, MICHAEL P. CAULFIELD, JOHN E. FISHER, MARK E. GOLDMAN, ROBERTA L. MCKEE, JANE E. REAGAN, JAY J. LEVY, RUTH F. NUTT, SEVGI B. RODAN, TIMOTHY L. SCHOFIELD, THOMAS L. CLEMENS, MICHAEL ROSENBLATT*

One mechanism considered responsible for the hypercalcemia that frequently accompanies malignancy is secretion by the tumor of a circulating factor that alters calcium metabolism. The structure of a tumor-secreted peptide was recently determined and found to be partially homologous to parathyroid hormone (PTH). The amino-terminal 1–34 region of the factor was synthesized and evaluated biologically. In vivo it produced hypercalcemia, acted on bone and kidney, and stimulated 1,25-dihydroxyvitamin D_3 formation. In vitro it interacted with PTH receptors and, in some systems, was more potent than PTH. These studies support a long-standing hypothesis regarding pathogenesis of malignancy-associated hypercalcemia.

THREE PRINCIPAL MECHANISMS have been proposed to account for the hypercalcemia of malignancy that occurs in humans and animals with certain tumors: direct osteolysis of bone by tumor metastases in direct contact with bone, resulting in release of calcium into the extracellular fluid; local production of bone-resorbing substances, a mechanism characteristic of hematologic and other malignancies; and tumor secretion of prostaglandins or growth factors or other peptides that circulate and act on bone or kidney (to decrease calcium excretion) in an endocrine fashion, even in the absence of bony metastases (*1*).

In 1941 Fuller Albright (*2*) proposed the last mechanism, namely, that tumors could secrete parathyroid hormone (PTH) ectopically and cause hypercalcemia. Subsequently, several studies demonstrated that a PTH-like factor, physicochemically and immunologically distinct from PTH, is secreted by tumor cells (*3*). Furthermore, messenger RNA for PTH was not found in tumors (*4*). However, the factor does stimulate adenylate cyclase in PTH target cells, and this activity can be inhibited by PTH antagonists (*3*). Three groups of investigators recently isolated and obtained partial amino acid sequences of peptide derived from several different human tumors (lung squamous carcinoma, renal cell carcinoma, and breast carcinoma) (*5, 6*). One group published the putative full-length peptide structure (141 amino acids) based on the complementary DNA (cDNA) nucleotide sequence (*7*). In each case, within the NH_2-terminal 13 residues there is considerable homology to the biologically critical NH_2-terminal region of PTH (*8, 9*) (Fig. 1), although the factor appears to be the product of a different gene (*7*).

In order (i) to determine if this human "humoral hypercalcemic factor" (hHCF) alone can, as implied, produce hypercalcemia and other components of the humoral hypercalcemia of malignancy (HHM) syndrome in vivo, (ii) to further define its actions in vitro, and (iii) to compare its biological profile and potency to those of PTH, we chemically synthesized an NH_2-terminal fragment of hHCF (*10, 11*).

Using the thyroparathyroidectomized rat as an assay system (*12*), we compared the activity of hHCF-(1–34)NH_2 with that of bovine PTH, bPTH-(1–84). In this system, hHCF-(1–34)NH_2 produced hypercalcemia with an apparent potency six to ten times as great as bPTH-(1–84) (Fig. 2) (*13*). The hypercalcemia could result from direct actions on bone or kidney, or via 1,25-dihydroxyvitamin D_3 action on gut, or any combination of these activities. Reductions in serum phosphate and elevations in circulating 1,25-dihydroxyvitamin D_3, which is the active form of vitamin D and is physiologically regulated by PTH, were also observed. The hHCF-(1–34)NH_2 also had other PTH-like effects on the kidney (*14*), increasing excretion of adenosine 3′,5′-monophosphate (cAMP) and phosphate. Again, hHCF-(1–34)NH_2 was more potent than bPTH-(1–84) (Fig. 3). When high doses were administered for 16 hours, a white precipitate was observed in the kidneys that presumably represented nephrocalcinosis; this was not seen with the doses of bPTH administered.

The action of hHCF-(1–34)NH_2 on vitamin D metabolism was particularly pronounced relative to bPTH and important with regard to its implication for promoting long-term hypercalcemia clinically. In addition to mobilizing calcium release from bone and having PTH-like actions on kidney, hHCF could contribute to HHM by increasing levels of 1,25-dihydroxyvitamin D_3 that then promote the gastrointestinal absorption of dietary calcium. Elevated levels of 1,25-dihydroxyvitamin D_3 are inconsistently found in human hyperparathyroidism and usually not found in HHM, perhaps because 1,25-dihydroxyvitamin D_3 production is suppressed by elevated calcium levels or because renal function is compromised in HHM (*15*). However, in animal models of HHM, including the transplantation of human tumor xenografts into

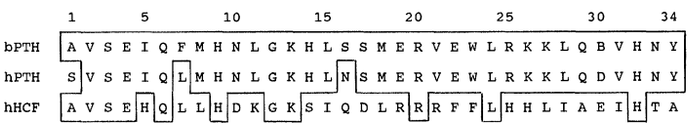

Table 1. Activity of hHCF-(1–34)NH_2 and PTH peptides in vitro. The values presented represent the mean of at least three experiments ± SEM. The K_b values were calculated as described (*21*). For binding studies, HPLC-purified mono-iodinated [Nle-8,Nle-18,Tyr-34]bPTH-(1–34)NH_2 was the radioligand.

Peptide	Renal membranes		Bone (ROS cells) adenylate cyclase K_m (n*M*)
	Binding K_b (n*M*)	Adenylate cyclase K_m (n*M*)	
hHCF-(1–34)NH_2	13.3 ± 2.6	36.6 ± 4.3	1.0 ± 0.1
bPTH-(1–84)	4.9 ± 0.2	14.3 ± 8.7	4.8 ± 0.1
bPTH-(1–34)	0.8 ± 0.2	1.8 ± 0.1	1.1 ± 0.1
hPTH-(1–84)	63.7 ± 19.4	116.0 ± 32.0	91.5 ± 30.5
hPTH-(1–34)	1.2 ± 0.2	5.4 ± 0.2	2.0 ± 0.5

N. Horiuchi and T. L. Clemens, Regional Bone Center, Helen Hayes Hospital (New York State Department of Health), West Haverstraw, NY 10993.
M. P. Caulfield, J. E. Fisher, M. E. Goldman, R. L. McKee, J. E. Reagan, J. J. Levy, R. F. Nutt, S. B. Rodan, T. L. Schofield, M. Rosenblatt, Department of Biological Research and Molecular Biology, Merck Sharp & Dohme Research Laboratories, West Point, PA 19486.

*To whom correspondence should be addressed.

nude mice (*16*), levels of 1,25-dihydroxyvitamin D_3 are consistently elevated.

To determine whether bone is a target organ for hHCF-(1–34)NH_2 and whether the action on bone per se can produce hypercalcemia, we used thyroparathyroidectomized rats fed a low calcium diet. The rats were also nephrectomized. The absence of the parathyroids and thyroid gland would eliminate the possibility of PTH-secretagogue activity by the factor or the possible opposing hypocalcemic action of calcitonin

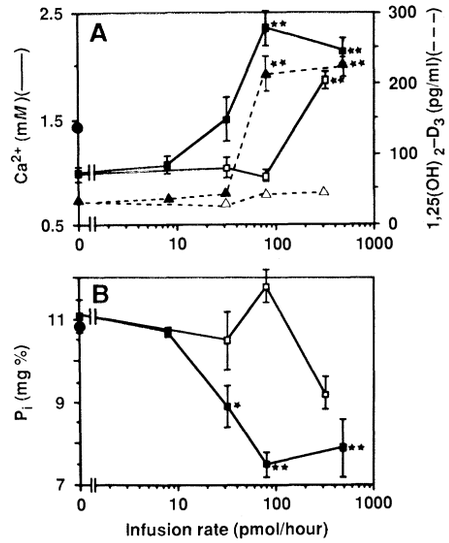

Fig. 2. Effects of bPTH-(1–84) (open symbols) and hHCF-(1–34)NH_2 (solid symbols) on (**A**) serum concentrations of ionized calcium (solid lines), 1,25-dihydroxyvitamin D_3 (broken lines), and (**B**) phosphate in thyroparathyroidectomized rats (*22*). Peptides or vehicle (in sham-treated animals) (●) were continuously infused (8 to 480 pmol/hour) into rats for 16 hours as described (*12*). Values represent the mean ± SEM for each group (n = 3 to 5); $^{*}P < 0.05$; $^{**}P < 0.01$ (*23*).

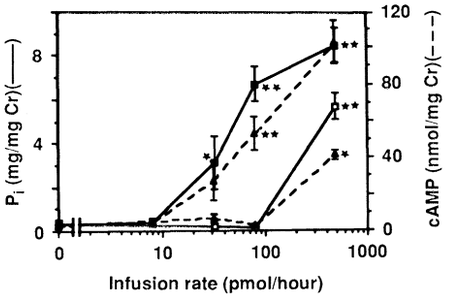

Fig. 3. Effects of bPTH-(1–84) (open symbols) and hHCF-(1–34)NH_2 (solid symbols) on urinary excretion of phosphate (solid lines) and cAMP (broken lines) with the assay (*12*) as described in the legend to Fig. 2, except that peptides were infused for 4 hours beginning 4 hours after thyroparathyroidectomy (*22*). The ordinates indicate average of urinary excretion of phosphate or cAMP per 30-minute interval (corrected for creatinine). Values represent the mean ± SEM for each group (n = 3 to 5). Values that are statistically different from control levels are indicated as in Fig. 2 (*23*).

secreted in response to rises in blood calcium levels. The low calcium diet reduces the contribution of dietary calcium; nephrectomy eliminates an action on alteration in renal clearance of calcium and minimizes the effect of generating 1,25-dihydroxyvitamin D_3 on overall calcium metabolism. In this assay, hypercalcemia would be of skeletal origin. The hHCF-(1–34)NH_2 increased calcium to an extent comparable with that obtained with bPTH-(1–34) and greater than that obtained with bPTH-(1–84) (Fig. 4). The finding that the effects of hHCF-(1–34)NH_2 and bPTH were comparable in this assay suggests that contributions from kidney or gut, or both, may account for the enhanced calcemic potency observed in the intact animal assay described above (*12*).

We also evaluated the interaction of hHCF-(1–34)NH_2 with PTH receptors in vitro. Binding affinity comparable to PTH for renal PTH receptors was observed (Table 1). In an adenylate cyclase assay in which we used bovine renal cortical membranes (*17*), hHCF-(1–34)NH_2 had a potency comparable to PTH. A close correspondence between renal binding (K_b) and activation constants (adenylate cyclase stimulation, K_m) was observed for all peptides tested (Table 1), although formal statistical comparison was not performed.

In another assay for adenylate cyclase, we used intact bone-derived osteosarcoma (ROS 17/2.8) cells and measured cAMP levels (*18*). The hHCF-(1–34)NH_2, unlike the bovine and human 1–34 and 1–84 PTH peptides, was more potent in stimulating bone than renal adenylate cyclase, a finding

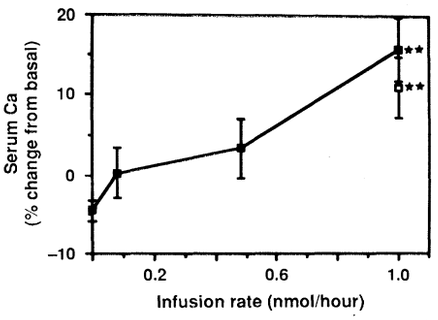

Fig. 4. Effects of bPTH-(1–34) and hHCF-(1–34)NH_2 on serum calcium in thyroparathyroidectomized nephrectomized rats. Male Sprague-Dawley rats (170 to 260 g) were thyroparathyroidectomized, and femoral vein cannulas were implanted (*22*). After surgery, rats were fed a low calcium diet. Bilateral nephrectomy was performed 16 hours later and peptide-containing solution or saline was infused. Blood was sampled immediately before and 3 hours after the start of infusion. bPTH-(1–34) (□); hHCF-(1–34)NH_2 (■). Baseline serum calcium (n = 65 rats) was 6.93 ± 0.2 mg % (mean ± SEM). Values represent the mean ± SEM for each group ($n \geq 8$). Values that are statistically different from control levels are indicated as in Fig. 2 (*23*).

similar to that observed by others using tumor extracts or conditioned media or other bone-derived cells (*5, 19*). The hHCF-(1–34)NH_2 was also approximately 100 times as potent as hPTH-(1–84) in this assay (Table 1).

These data with bone cells may explain, in part, the enhanced calcemic response to hHCF-(1–34)NH_2 versus bPTH-(1–84) in vivo. Alternatively, the finding that PTH receptor affinity is closely similar for hHCF-(1–34)NH_2 and several forms of PTH suggests that hHCF-(1–34)NH_2 might possess increased stability in vivo. The increased potency of hHCF-(1–34)NH_2 relative to PTH may also indicate differences between bone and renal PTH receptors or differences in receptor coupling to second messenger systems, or even the presence of a new class of receptors for hHCF with which both PTH and hHCF might interact.

This investigation addresses a theoretical concept of tumor pathogenesis proposed over 40 years ago, namely, that malignancy-associated hypercalcemia can result from endocrine secretion and action of a PTH-like factor by tumors. We have demonstrated that a fragment of hHCF alone, without added cofactors or hormones, can produce hypercalcemia and other biochemical abnormalities associated with HHM. The hypercalcemia can be generated by hHCF-(1–34)NH_2 action on bone, although kidney and gut could contribute to the HHM syndrome when it occurs naturally. Although the greatest homology of hHCF-(1–34)NH_2 to PTH occurs within the NH_2-terminal 13 positions, the biological profile of hHCF-(1–34)NH_2 is closely similar to PTH, implicating the importance of these residues in expression of calcium metabolism bioactivity. That hHCF-(1–34)NH_2 is more potent than PTH in some systems is of considerable interest for the future design of hormone analogs. Finally, no other tumor-secreted peptide displays this biological profile. This study establishes one (PTH-like) mechanism by which human tumors can produce hypercalcemia and suggests the potential utility of PTH antagonists (*9, 20*) in treatment.

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23. Statistically significant differences were observed among groups by one-way analysis of variance ($P < 0.01$) in all parameters measured. Values that are significantly different from control are indicated by * ($P < 0.5$) or *($P < 0.01$) [C. W. Dunnett, *J. Am. Stat. Assoc.* **50**, 1096 (1955)].
24. We are grateful to M. Chorev for critical reading of the manuscript, M. Buddle for technical assistance, S. Camburn for secretarial assistance, and the Audio-Visual Department of Helen Hayes Hospital. This work was supported, in part, by NIH grants AR 36446 and AR 39191 (T.L.C.) and Merck Sharp & Dohme Research Laboratories.

28 August 1987; accepted 22 October 1987

Parathyroid Hormone-Related Protein of Malignancy: Active Synthetic Fragments

BRUCE E. KEMP, JANE M. MOSELEY, CHRISTINE P. RODDA, PETER R. EBELING, RICHARD E. H. WETTENHALL, DAVID STAPLETON, HANNELORE DIEFENBACH-JAGGER, FIONA URE, VALDO P. MICHELANGELI, HOLLIS A. SIMMONS, LAWRENCE G. RAISZ, T. JOHN MARTIN

Peptides corresponding to the amino-terminal region of the parathyroid hormone–related protein (PTHrP) of humoral hypercalcemia of malignancy were synthesized. A 34–amino acid peptide, PTHrP(1–34), was two to four times more potent than bovine or human PTH(1–34) in bioassays promoting the formation of adenosine 3′,5′-monophosphate (cAMP) and plasminogen activator activity in osteogenic sarcoma cells and adenylate cyclase activity in chick kidney membranes. Like parathyroid hormone itself, in which the activity resides in the first 34 residues, PTHrP peptides of less than 30 residues from the amino terminus showed substantially reduced activity. PTHrP(1–34) had only 6% of the potency of bovine PTH(1–34) in promoting bone resorption in vitro. PTHrP(1–34) strongly promoted the excretion of cAMP and phosphorus and reduced the excretion of calcium in the isolated, perfused rat kidney consistent with the symptoms seen in malignant hypercalcemia.

STUDIES OF HUMORAL HYPERCALCEMIA of malignancy (HHM) have provided evidence that tumors produce a protein that acts through the parathyroid hormone (PTH) receptor but is immunologically distinct from PTH (*1*). A PTH-related protein (PTHrP) was isolated from a lung cancer cell line, and 8 of its 16 amino-terminal residues were identical with human PTH (*2*). The amino acid sequence to residue 50 was subsequently determined and a full-length complementary DNA encoding a 141–amino acid protein was isolated (*3*). The homology with PTH is restricted to the amino-terminal region. Since the biological activity of the 84–amino acid residue PTH molecule is contained within the first 34 residues (*4*), we investigated the biological actions of synthetic peptide analogs of the amino-terminal sequence of PTHrP (*3*). We found that PTHrP(1–34) has substantial biological activity in five PTH response systems, and that PTHrP peptides of chain length less than 30 residues have greatly attenuated activities.

The activity of the synthesized and purified PTHrP(1–34) (*5*) was studied in the PTH-responsive osteogenic sarcoma cell line UMR 106-01 (*6*). PTHrP(1–34) stimulated adenosine 3′,5′-monophosphate (cAMP) formation in a dose-dependent manner with a potency four times greater than that of either human or bovine PTH(1–34); the parent protein is six times more potent than bovine PTH(1–34) in the same assay system (*2*). PTHrP(1–34) was approximately equal in potency to rat PTH(1–34) (Fig. 1A) which is several times more potent than either the human or bovine PTH(1–34) (*7*). Similar relative potencies of PTHrP(1–34) and PTH(1–34) were evident in bioassays based on measurements of the activities of membrane adenylate cyclase from chicken kidney.

We also synthesized a series of shorter PTHrP peptides. These were appreciably less potent in the osteogenic sarcoma cell cAMP response (Fig. 1B). Synthetic PTHrP(1–29) had about 10% of the activity of PTHrP(1–25) and less than 0.01% of the activity of PThrP(1–34). There was no measurable activity in the shorter peptides, PTHrP(1–20) and PTHrP(1–14). Virtually identical relative activities were obtained in the chicken kidney adenylate cyclase response. Thus the region of PTHrP responsible for its known biological activities is contained within the amino-terminal 34 residues, a situation analogous to that with PTH (*4*).

PTHrP(1–34) was also more potent than PTH(1–34) in stimulating tissue plasminogen activator (tPA) activity produced by osteogenic sarcoma cells (*8*). In contrast, the relative potencies in promoting bone resorption in vitro were reversed. The effects of PTHrP(1–34) and bovine PTH(1–34) on bone resorption were assayed as the release of previously incorporated ^{45}Ca from cul-

B. E. Kemp, J. M. Moseley, C. P. Rodda, P. R. Ebeling, D. Stapleton, H. Diefenbach-Jagger, F. Ure, V. P. Michelangeli, T. J. Martin, University of Melbourne, Department of Medicine, Repatriation General Hospital, Heidelberg, Victoria, Australia 3081.
R. E. H. Wettenhall, La Trobe University, Department of Biochemistry, Bundoora, Victoria, Australia 3083.
H. A. Simmons and L. G. Raisz, Division of Endocrinology, University of Connecticut Health Sciences Center, Farmington, CT.