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A Parathyroid Hormone–Like Protein from Cultured Human Keratinocytes

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Parathyroid hormone–like factors have been found in extracts of tumors associated with humoral hypercalcemia of malignancy, many of which are of squamous epithelial origin. Cultured, nonmalignant human keratinocytes were examined for the production of similar factors. Keratinocyte-conditioned medium from ten cultures stimulated the production of cyclic adenosine monophosphate in clonally derived rat osteosarcoma cells sensitive to parathyroid hormone. Bovine [Nle^{8,18}, Tyr^{34}]PTH-(3-34)NH_2, a competitive inhibitor of parathyroid hormone, stopped the adenylate cyclase production stimulated by keratinocyte-conditioned medium, but antisera to parathyroid hormone had no effect on such adenylate cyclase activity. The active component of keratinocyte-conditioned medium has a molecular weight exceeding that of native parathyroid hormone. These characteristics are shared by the parathyroid hormone receptor agonists associated with humoral hypercalcemia of malignancy, which suggests that normal human keratinocytes may produce a factor related to that produced by malignant tumors associated with humoral hypercalcemia of malignancy.

HYPERCALCEMIA IS THE MOST common paraneoplastic syndrome and affects up to 40 percent of patients with certain types of cancer (*1*). Although some cases result from direct destruction of bone by metastatic tumor, hypercalcemia frequently arises in patients without skeletal involvement (*1*). There is substantial evidence that in such instances the elevated serum calcium results from the secretion by tumors of a circulating factor (or factors) that leads to bone resorption (*1–3*). In one series of 50 unselected patients with cancer and hypercalcemia, nearly 80 percent suffered from this humoral hypercalcemia of malignancy (HHM) (*2*).

Certain factors derived from tumors associated with HHM share functional properties with parathyroid hormone (PTH). For example, we reported that extracts of four of five tumors from patients with HHM stimulated the PTH-sensitive adenylate cyclase in canine renal cortical membranes, whereas extracts of tumors from patients without HHM did not (*4*). This activity was competitively inhibited by PTH receptor antagonists but not by an antiserum directed against PTH. Similar findings have been reported by Strewler and co-workers (*5*) who used an HHM-associated human renal carcinoma cell line. In addition, Rodan *et al.* (*6*) demonstrated that medium conditioned by cells derived from HHM-associated tumors stimulated formation of cyclic adenosine monophosphate (AMP) by PTH-sensitive rat osteosarcoma (ROS) cells in a dose-dependent fashion (*6*). This activity was related to bone resorption in vitro.

Tumors with squamous epithelial features, including those of the oropharynx, lung, esophagus, cervix, vulva, and skin, account for the largest single subset of HHM-associated tumors (*1, 2*). We therefore examined nonmalignant human keratinocytes to determine whether nonneoplastic squamous epithelial cells might produce a factor (or factors) similar to the PTH receptor agonists derived from HHM-associated tumors. Our findings indicate that cultured human keratinocytes do produce an adenylate cyclase–stimulating protein that acts through the PTH receptor but that is distinct from native PTH.

Cultures of human keratinocytes were established from ten neonatal human foreskins (*7*). First passage keratinocytes were grown 1 week past confluence either in dishes containing irradiated murine 3T3 fibroblasts in complete medium [Dulbecco's modified Eagles medium (DMEM) containing fetal bovine serum (20 percent; KC Biologicals), hydrocortisone (0.4 μg/ml), and antibiotics (*7*)] or in dishes coated with type I collagen (Collagen Corp.) in calcium-free DMEM containing fetal bovine serum (10 percent), hydrocortisone (0.4 μg/ml), and antibiotics (*8*).

Keratinocyte-conditioned medium (KCM) harvested from confluent, first-passage cultures 48 hours after medium change was tested for parathyroid hormone–like bioactivity in the ROS assay with a modification of the method of Rodan and co-workers (*6, 9*). This assay measures cyclic AMP production (as an index of adenylate cyclase activity) by clonal ROS 17/2.8 cells in response to PTH receptor agonists. The assay has a detection limit of $5 \times 10^{-11}M$ PTH and shows a linear response over the range of 1×10^{-10} to $2.5 \times 10^{-9}M$. Normal PTH is believed to circulate at 10^{-12} to $10^{-11}M$. The only other known agonists in this system are β-adrenergic agonists such as isoproterenol.

Conditioned medium from each of the ten keratinocyte cultures stimulated adenylate cyclase activity in the ROS assay (Table 1). Four cultures of irradiated 3T3 cells, three cultures of neonatal foreskin dermal

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fibroblasts, and one culture of human epidermal melanocytes, each maintained in the same medium as the keratinocytes, served as controls. Results are given as counts per minute (cpm) of ^{3}H-labeled cyclic AMP per well. The results indicate that medium from each of the ten keratinocyte cultures possessed substantial adenylate cyclase–stimulating activity, generating from 8170 to 31,280 cpm per well, compared with a mean basal value of 1420. None of the conditioned medium samples from the control cultures generated a significant increase in cyclic [^{3}H]AMP production.

The effect of KCM on the ROS cells appears to be mediated through the PTH receptor. Figure 1A illustrates the ROS cell dose-response curves for bovine PTH-(1–34), isoproterenol, and a typical sample of KCM, all tested in a single assay. KCM yields a curve indistinguishable from that of bovine PTH-(1–34), whereas the curve from isoproterenol has a slope nearly twice as great. Figure 1B shows that co-incubating KCM with increasing concentrations of bovine [Nle^{8,18},Tyr^{34}]PTH-(3–34)NH_2, a synthetic PTH receptor antagonist, progressively and completely inhibits the formation of cyclic [^{3}H]AMP. These results support the hypothesis that a component of KCM stimulates the ROS cell adenylate cyclase by binding to the PTH receptor.

Antibody inhibition studies, however, indicate that the KCM adenylate cyclase–stimulating activity is not identical to that of PTH. Prior incubation of $7.5 \times 10^{-10}M$ bovine PTH-(1–34) diluted in complete medium for 1 hour at 27°C with a 1:160 dilution of G-5 [a goat antiserum to human PTH that reacts predominantly with the midregion of the PTH molecule (*10*)] or with a 1:1000 dilution of CK-67 [a chicken antiserum to human PTH that reacts predominantly with the amino terminal region of the PTH molecule (*11*)] reduced cyclic AMP production by ROS cells from 6460 cpm per well to 890 or 1060 cpm per well, respectively. The basal value in this assay was 1210 cpm per well. In contrast, prior incubation of KCM with identical dilutions of the same two antisera resulted in no decrease in cyclic AMP production: a sample of KCM yielded 4200 cpm per well, whereas KCM incubated with G-5 or with CK-67 under conditions identical to those described above yielded values of 3920 or 4630 cpm per well, respectively. These results were confirmed in two additional experiments.

Studies on the physical characteristics of the active component of KCM indicate that it is a protein. KCM was treated with trypsin (0.25 g/liter) at 27°C for 30 minutes. The trypsin was then inactivated with soybean trypsin inhibitor (0.5 g/liter at 27°C for 30 minutes). This protease treatment completely eliminated the adenylate cyclase–stimulating activity in KCM. The activity was stable to heating (100°C for 10 minutes) and to treatment with 500 mM acetic acid. The adenylate cyclase–stimulating activity eluted from a 0.9 by 50 cm gel filtra-

Table 1. Cyclic AMP production by rat osteosarcoma cells after exposure to conditioned medium from keratinocyte and control cultures. Conditioned medium from ten confluent cultures of keratinocytes grown either on a feeder layer of murine 3T3 fibroblasts or on collagen was assayed with the rat osteosarcoma adenylate cyclase assay and compared with medium conditioned by cultures of human dermal fibroblasts, of murine 3T3 fibroblasts, and of human melanocytes. Basal (unstimulated) adenylate cyclase activity was 1420 cpm per well. The range of adenylate cyclase activity induced by the KCM cultures (8170 to 31,280 cpm per well) is equivalent to that produced by 150 to 1000 pM bovine PTH-(1–34).

Medium sample	Cyclic [^{3}H]AMP (cpm per well)
Keratinocytes on 3T3 cells	
KCM 1	11,660
KCM 2	8,170
KCM 3	31,280
KCM 4	12,600
KCM 5	11,010
KCM 6	10,520
KCM 7	17,150
KCM 8	9,400
Keratinocytes on collagen	
KCM 9	18,650
KCM 10	16,420
Murine 3T3 fibroblasts	
3T3 1	3,000
3T3 2	1,440
3T3 3	1,320
3T3 4	1,020
Dermal fibroblasts	
DF 1	740
DF 2	2,350
DF 3	1,620
Melanocytes	
MEL 1	910
Basal	
Complete medium	1,420

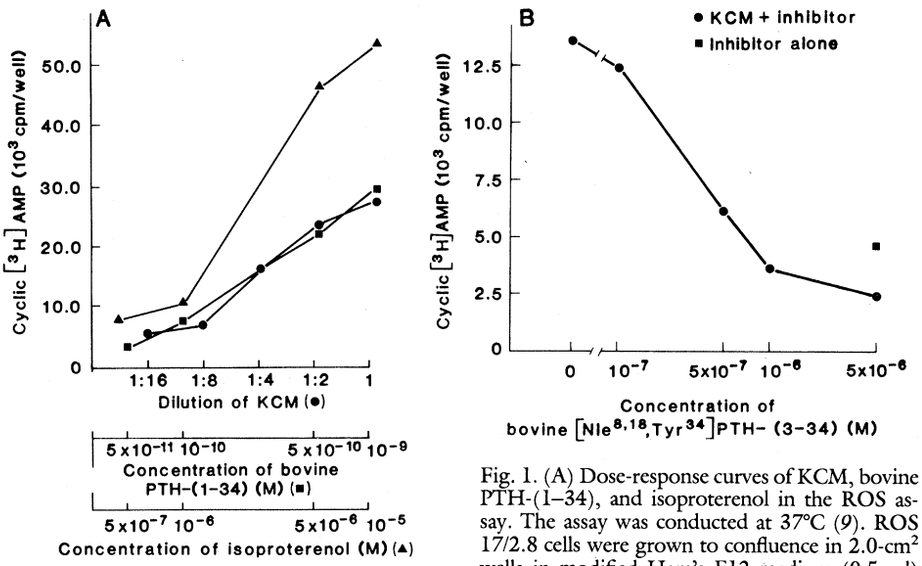

Fig. 1. (A) Dose-response curves of KCM, bovine PTH-(1–34), and isoproterenol in the ROS assay. The assay was conducted at 37°C (*9*). ROS 17/2.8 cells were grown to confluence in 2.0-cm^2 wells in modified Ham's F12 medium (0.5 ml) with fetal bovine serum (5 percent), penicillin (100 U/ml), and streptomycin (100 μg/ml). On the day of assay, [^{3}H]adenine (2 μCi) in water (20 μl) was added to each well, and the incubation was continued at 37°C for exactly 2 hours. The medium was then aspirated, and the cells were washed twice with calcium- and magnesium-free Hanks buffered saline (0.5 ml; Grand Island Biological). The cells were then incubated for 10 minutes with Ham's F12 medium (100 μl) containing fetal bovine serum (2 percent) and isobutylmethylxanthine (400 μM; Sigma). KCM or standard [bovine PTH-(1–34) or isoproterenol diluted in complete medium (100 μl)] was then added to appropriate wells. Incubations were terminated after 5 minutes for bovine PTH-(1–34) and after 10 minutes for KCM or isoproterenol (*6, 9*) by adding ice cold 1.2M trichloroacetic acid (20 μl). A carrier solution (100 μl) containing 5 mM each of cyclic AMP, adenine, adenosine, AMP, ADP, and ATP was then added to each well. Forty μl of water containing 2000 cpm of cyclic [^{32}P]AMP was next added to each well as a recovery standard. The reaction mixture was frozen at −20°C, thawed, centrifuged at 4500*g* for 10 minutes, and neutralized with 4N KOH. The supernatants were collected, and radioactively labeled cyclic AMP was separated by serial elution on ion exchange and alumina columns. Samples were counted by liquid scintillation, and the counts were corrected for recovery. Each sample was assayed in duplicate. All duplicate values shown in the figures and Table 1 were within 10 percent of one another. Complete medium assayed alone yielded the basal value. (B) Effect of co-incubation with increasing concentrations of bovine [Nle^{8,18},Tyr^{34}]PTH-(3–34)NH_2 on the adenylate cyclase–stimulating activity of KCM. This antagonist did not influence the adenylate cyclase–stimulating activity induced by isoproterenol. The basal value in this assay was 850 cpm per well.

tion column (Sephadex G-75) developed in 100 m*M* acetic acid (*p*H 3.0), in a discrete peak with an apparent molecular weight in the range of 25,000 to 40,000 daltons, well ahead of native bovine PTH-(1–84) (molecular weight, 9100).

Our studies indicate that cultured human keratinocytes produce a PTH-like factor that stimulates cyclic AMP formation in cultured rat osteosarcoma cells but differs from PTH both immunologically and with respect to apparent molecular weight. PTH receptor agonists derived from HHM-associated tumors share these characteristics (*4–6*), which suggests that such tumors may secrete a factor related to that produced by certain normal squamous epithelia, such as the skin. Further studies are necessary to determine whether the active component of KCM possesses bone-resorbing activity (the cardinal feature of any putative HHM factor) and to determine the precise structural relation between the keratinocyte- and tumor-derived adenylate cyclase–stimulating factors.

The determination of a possible physiologic role for this factor must also await further investigation. Although the dermis is not traditionally regarded as a PTH target organ, recent studies have demonstrated the presence of receptors that recognize both PTH and the putative HHM-factor on dermal fibroblasts (*12*). This suggests that the PTH-like substance produced by keratinocytes may play a role in normal skin physiology.

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Protein-Tyrosine Kinase Activity in *Saccharomyces cerevisiae*

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***Saccharomyces cerevisiae* was examined for tyrosine kinase activity in vitro because this organism offers molecular and genetic approaches for analyzing the role of tyrosine phosphorylation in cellular growth control that are unavailable in higher eukaryotes. Yeast extracts phosphorylated a random copolymer (glutamic acid:tyrosine, 80:20) at tyrosine in a reaction that was linear with respect to time and protein concentration. In the absence of added copolymer, phosphotyrosine was 0.1 percent of the total phosphoamino acids labeled with [γ-^{32}P]adenosine triphosphate in endogenous yeast proteins. However, specific activities of these reactions were low (approximately 1 percent of those in extracts of chick embryo fibroblasts). Lack of significant incorporation of label from [α-^{32}P]adenosine triphosphate into the copolymer or endogenous yeast proteins demonstrated that nucleotide interconversion, adenylylation, and subsequent hydrolysis could not account for the generation of phosphotyrosine observed.**

PROTEIN PHOSPHORYLATION AT TYROSINE appears to be important in growth control and in malignant transformation of animal cells (*1*). A variety of cellular protein substrates for these enzymes have been identified (*2*), but the function of these target proteins in growth control or in oncogenesis remains unclear. Yeast is a eukaryote that provides several advantages for the study of growth regulation, including its short generation time, small genome size, the availability of many mutations that affect the cell division cycle (*3*), and facile application of recombinant DNA methods, such as gene replacement (*4*). Yeast has already proven valuable for investigating the function of the *ras* family of proto-oncogenes (*5, 6*). We describe here the use of a sensitive assay to determine if yeast extracts contain tyrosine kinase activity.

Braun *et al.* (*7*) first used a synthetic random copolymer of 80 percent glutamic acid and 20 percent tyrosine [poly(Glu^{80}Tyr^{20})] and [γ-^{32}P]adenosine triphosphate (ATP) for the assay of several purified tyrosine kinases. Our initial experiments with crude yeast extracts indicated that the presence of endogenous phosphoacceptor proteins made precipitation with acid (*7, 8*) an ineffective single method for measuring specific incorporation into the polymer. Hence we sought an alternative method for separating the phosphorylated polymer from the other reaction components. Although poly (Glu^{80}Tyr^{20}) is heterogeneous in size (molecular weight, 20K to 60K), all the species present have the same charge-to-mass ratio because of their constant composition. For this reason and because of their strongly negative net charge, we anticipated that the population of molecules should migrate rapidly as a relatively discrete band when subjected to electrophoresis in polyacrylamide gels in the absence of any ionic detergent, provided that the matrix imposes little sieving action. We were able to find conditions (Fig. 1) under which poly(Glu^{80}Tyr^{20}) migrated as a doublet just slightly slower than the dye front, as revealed by staining with Coomassie blue.

To test the feasibility of this approach as an assay procedure for tyrosine kinase activity, extracts were prepared from uninfected

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