Identification of Transcripts Encoding a Parathyroid Hormone-like Peptide in Messenger RNAs from a Variety of Human and Animal Tumors Associated with Humoral Hypercalcemia of Malignancy

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Abstract

The syndrome of humoral hypercalcemia of malignancy (HHM) appears to be mediated in many instances by a parathyroid hormone-like peptide, which has recently been purified, sequenced, and cloned. Using a probe representing the coding region of the human PTH-like peptide, we examined by Northern analysis poly $(A)^+$ RNA from a variety of human and animal tumors associated with HHM. Hybridizing transcripts were identified in mRNA from each of 12 human and each of four animal HHM-associated tumors, with a complex hybridization pattern observed in the human mRNAs and a relatively simple pattern observed in the animal mRNAs. Poly $(A)^+$ RNA prepared from tumors of similar histological types unassociated with HHM failed to hybridize with the probe. Messenger RNA-dependent biological activity from the animal tumors was entirely eliminated in a hybridization-arrest experiment using a complementary oligonucleotide spanning the region of homology between human PTH and the PTH-like peptide. These findings indicate that the PTH-like peptide is associated with the syndrome of HHM in a wide spectrum of tumor types from a variety of mammalian species and that the PTH-like sequence in the proximal amino terminus of the peptide is highly conserved.

Introduction

Humoral hypercalcemia of malignancy (HHM)[1] is a common paraneoplastic syndrome. Studies over the past decade have increasingly focused on a tumor-derived peptide with certain PTH-like actions as a frequent mediator of this syndrome (1–5). Although effects resembling those of PTH have been demonstrated both in vivo and in vitro, based on other of its actions, its size, and its immunoreactivity, the tumor-derived peptide is clearly distinct from native parathyroid hormone (2, 4, 5–9).

Three laboratories have recently purified PTH-like peptides from four different human tumors and generated partial amino-terminal sequences that appear to be identical and resemble the proximal amino-terminal sequence of human PTH (6–9). Two laboratories have also identified cDNA clones encoding the PTH-like peptide (10, 11). The peptide consists of a 36 amino-acid precursor sequence and a 141 amino-acid mature peptide (10, 11). Sequence similarity to human PTH is confined to the last two amino acids of the precursor sequence and the first 13 amino acids of the mature peptide. The gene for the PTH-like peptide appears to be a single-copy gene and has been mapped to chromosome 12, whereas the gene for PTH is located on chromosome 11 (11).

Our initial examination of poly $(A)^+$ RNAs prepared from several human HHM-associated tumors demonstrated a complex hybridization pattern, and it was suggested that the multiple mRNA species observed were likely the result of alternative RNA processing (11). We report here a larger analysis of mRNAs prepared from human and animal tumors associated with HHM as well as from similar tumor types unassociated with this syndrome. It was our intention in this larger analysis to (*a*) compare hybridization patterns across a representative sample of HHM-associated tumors, (*b*) determine whether a typical or reproducible hybridization pattern characterizes multiple examples of a single cell type (e.g., renal carcinomas), (*c*) examine certain biologically unusual examples of HHM (e.g., pheochromocytoma), and (*d*) examine several spontaneous (12–14) or man-made (15) HHM-associated tumors in subhuman mammalian species. In addition to Northern analysis, the animal-tumor mRNAs were studied using a hybridization-arrest protocol, in which a complementary oligonucleotide targeted at the region of homology between human PTH and the human PTH-like peptide was employed.

Methods

Tumors. The human HHM-associated tumors included five renal carcinomas (Sloan-Kettering Research Center Nos. 1, 8, 10, 38, and 52)

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Received for publication 12 January 1988 and in revised form 24 February 1988.

1. *Abbreviations used in this paper:* HHM, humoral hypercalcemia of malignancy.

J. Clin. Invest.

0021-9738/88/06/2010/05 $2.00
Volume 81, June 1988, 2010–2014

(3), two breast carcinomas (6, 7, 16), three squamous carcinomas (7, 17, 18), and two pheochromocytomas (17, 19). Three of these tumors were surgical specimens, three were autopsy specimens, and six (five renal carcinomas and one squamous carcinoma) were grown as xenografts in nude mice, in which they produced hypercalcemia (3, 11). Each of these 12 tumors has been shown to be associated with biochemical evidence of HHM in patients and/or nude mice in vivo (3, 6, 7, 16–19), and PTH-like bioactivity has been demonstrated in vitro in extracts and/or conditioned medium from each of the tumors and/or cultured lines (4–7, 16–19). Peptides from two of the tumors (one breast carcinoma and one squamous carcinoma) have been purified to homogeneity and partially sequenced (7). Renal carcinoma line SKRC-1 was the source of the mRNA used for construction of the cDNA library from which the clone was isolated (11). Both pheochromocytomas were clinically benign, in that excision of localized lesions resulted in complete cure (17, 19). The human control tumors included (*a*) eight renal carcinoma lines (SKRC Nos. 17, 21, 29, 31, 42, 49, 53, and line C) which do not secrete PTH-like activity in vitro and which do not (in the six instances tested) induce hypercalcemia when implanted in athymic mice (3) and (*b*) a lymphoma (surgical specimen) which appeared to induce hypercalcemia by producing 1,25-dihydroxyvitamin D (20).

The animal HHM-associated tumors included the transplantable H500 Leydig cell tumor in the Fisher rat (12), a carcinogen-induced squamous cell carcinoma in the mouse (15), and two spontaneous tumors in the dog, a lymphosarcoma (13) and an apocrine cell carcinoma (14). Each of these tumors is associated with biochemical evidence of HHM in vivo (12–15), and extracts of each tumor have been shown to contain PTH-like bioactivity in vitro (12, 13, 15).[2] The control tumors included the H540 Leydig cell line in the rat (21)[3] and a dog lymphosarcoma unassociated with hypercalcemia (13). All human and animal tumors were stored at −70°C until use.

Preparation of poly (A)$^+$ RNA. Total RNA was prepared from powdered frozen tissue using a modification of guanidinium thiocyanate-cesium chloride technique (22), and poly (A)$^+$ RNA was selected by oligo (dT)-cellulose chromatography. The integrity of the preparations of poly (A)$^+$ RNA was verified by translation in a nuclease-treated reticulocyte lysate (23); preparations that produced less than a twofold stimulation of [^{35}S]methionine incorporation into trichloroacetic acid-precipitable proteins were not further studied.

Northern blot analysis. Poly (A)$^+$ RNA was electrophoresed on agarose-formaldehyde gels and transferred to nylon filters (Hybond N; Amersham Corp., Arlington Heights, IL), essentially as described (24). Blots were prehybridized and then hybridized overnight with a ^{32}P-labeled antisense RNA probe (0.5×10^6 cpm/ml) at 70°C in a solution containing 50% formamide, 5× SSC, 0.5% SDS, 1× Denhardt's solution (0.01% each bovine serum albumin, polyvinylpyrolidine and Ficoll), and denatured, sheared salmon sperm DNA (100 μg/ml). The filters were washed in 0.1× SSC/0.1% SDS at 74°C for 1 h and exposed to Kodak XAR-5 film at −70°C for the indicated times. The probe used represents the coding region for the PTH-like peptide and is a restriction fragment containing 133 base pairs (bp) of 5′ untranslated sequence and the coding region through amino acid 137 of the deduced sequence of the mature peptide (11). This fragment was subcloned into pGEM-3-blue (Promega Biotech, Madison, WI), and the anti-sense RNA was synthesized using SP6 RNA polymerase and α[^{32}P]UTP (410 Ci/mmol; Amersham Corp.), as described by the supplier with modifications (25).

Hybridization-arrest, oocyte injection, and cytochemical bioassay. A 45-base oligonucleotide complementary to the sequence encoding amino acids −2 to +13 of the PTH-like peptide (see Fig. 4 in the text) was synthesized by the phosphoramidite method and purified on a denaturing polyacrylamide gel. This sequence represents the region of homology between human PTH and the human PTH-like peptide (67% sequence identity at both a nucleotide and amino-acid level) (11). In the hybridization arrest experiment, mRNAs (1 μg/μl) prepared from the animal tumors were incubated with and without this oligonucleotide (10 pmol/μl) prior to injection into oocytes, following the protocol described by Kawasaki except that NaCl was used at a final concentration of 100 mM (26). Xenopus oocytes were injected with 50 nl of the samples and media harvested and pooled after 36 h of incubation at 20°C (27). The media were assayed in the cytochemical bioassay for PTH (28), with results expressed in equivalence units (picogram equivalents per milliliter) to human PTH (1-84). A 45-base sense oligonucleotide spanning the same region (see Fig. 4) was also synthesized and used as a negative control with mRNA from line SKRC-1, employing concentrations and conditions identical to those described above.

Results

Human tumors. Poly (A)$^+$ RNA was prepared from a total of 21 human tumors. 12 of these were HHM-associated tumors; eight were renal carcinomas unassociated with HHM, and one was a lymphoma associated with 1,25-dihydroxyvitamin D-mediated hypercalcemia.

Renal carcinoma line SKRC-1 was taken as a frame of reference, in that the cDNA clone was isolated from a library prepared from this line (11), and multiple preparations of mRNA from this tumor have consistently revealed a pattern of five transcripts on Northern blots. These transcripts include two major hybridizing species of about 1.6 and 2.1 kilobases (kb) and three less abundant species of ~ 3.2, 3.8, and 4.2 kb (lane *1* in Figs. 1–3). Based on a comparison of known amounts of sense transcripts synthesized in vitro and added to control poly (A)$^+$ RNA as carrier and run on the gels (data not shown), the major transcripts in this line represent ~ 0.005% mRNA species and the minor transcripts ~ 0.001% mRNA species.

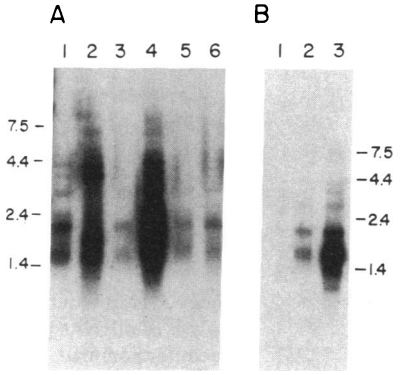

Figure 1. Northern blot analysis of human tumor-derived mRNAs. (*A*) Renal carcinoma line SKRC-1 (lane *1*, 4 μg), breast carcinoma from which amino-terminal peptide sequence was obtained (7) (lane *2*, 3 μg), breast carcinoma (lane *3*, 8 μg), squamous carcinoma grown in nude mice (lane *4*, 4 μg), squamous carcinoma (lane *5*, 8 μg), and squamous carcinoma from which amino-terminal peptide sequence was obtained (7) (lane *6*, 6 μg). (*B*) Lymphoma associated with 1,25-dihydroxyvitamin D production (20) (lane *1*, 4 μg) and pheochromocytomas (lanes *2* and *3*, 4 μg each). Note that A and B are from separate gels; the hybridizing bands in mRNA from line SKRC-1 and the pheochromocytoma shown in lane *3* of B co-migrated precisely when run on the same gel. The autoradiogram is a 24-h exposure, and the markers correspond to the RNA ladder from Bethesda Research Laboratories.

2. Weir, E. C., et al., manuscript submitted.

3. The H540 line was obtained from the Mason Research Institute Tumor Bank (Worcester, MA). This tumor is not associated with hypercalcemia when maintained in Wistar rats for up to three months, and tumor extracts are devoid of PTH-like bioactivity in vitro (K. L. Insogna, unpublished observations).

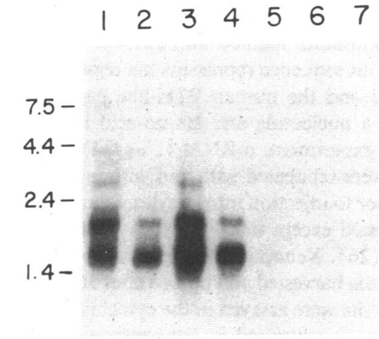

Figure 2. Northern blot analysis of mRNAs prepared from a series of human renal carcinomas. The first four lanes contained mRNA from HHM-associated renal carcinomas (lines SKRC-1, 52, 10, and 38, respectively). Lanes *5–7* contained mRNA from renal carcinomas that do not secrete PTH-like activity in vitro and that also do not induce hypercalcemia when grown as xenografts in nude mice (3) (lines SKRC-31, 42, and 17, respectively). Poly $(A)^+$ RNA preparations from five additional control renal carcinomas failed to hybridize with the probe. The autoradiogram is a 12-h exposure, and each mRNA was run at 4 μg/lane.

Multiple hybridizing transcripts were observed in mRNA from each of the 12 HHM-associated tumors. The ~ 1.6- and 2.1-kb species were consistently observed, and a majority of the mRNAs also contained either two or three of the transcripts in the 3.2- to 4.2-kb range (Figs. 1 and 2). Shorter exposures revealed major transcripts of 1.6 and 1.8 (rather than 2.1) kb in mRNA from one of the breast carcinomas (lane *2* in *A* of Fig. 1) and a trio of major transcripts of ~ 1.8, 1.9, and 2.1 kb in the mRNA from one of the squamous carcinomas, which projected as somewhat of a smear on all but the shortest exposures (lane *4* in *A* of Fig. 1). The hybridization pattern observed in mRNA from one of the pheochromocytomas (lane *3* in *B* of Fig. 1) was identical to that observed in line SKRC-1 mRNA, a point of some interest given the apparently benign nature of the pheochromocytoma. The faintest and least complex hybridization patterns were observed in three mRNA preparations that did not translate well (mean twofold stimulation of $[^{35}S]$methionine incorporation) and which were presumably partially degraded (two were autopsy specimens and one a surgical specimen).

To determine whether a reproducible hybridization pattern characterizes multiple examples of a single tumor type, mRNA preparations from five human renal carcinomas were examined. The hybridization pattern observed in these mRNAs was essentially identical (four are shown in Fig. 2; the fifth is not shown). Two of these mRNAs contained all five transcripts, two all but the 4.2-kb transcript, and one all but the 3.8- and 4.2-kb transcripts. This hybridization pattern was not unique to mRNAs from renal carcinomas but was in general quite similar to that observed in mRNAs from other tumor types.

None of the mRNA preparations from the nine tumors unassociated with HHM hybridized with the probe. These included eight renal carcinomas (three of which are shown in lanes *5–7* of Fig. 2) and a lymphoma associated with 1,25-dihydroxyvitamin D production (lane *1* of *B*, Fig. 1).

Animal tumors. Northern analysis of the mRNAs prepared from the six animal tumors is shown in Fig. 3. One or more hybridizing transcripts was observed in poly $(A)^+$ RNA from each of the four HHM-associated tumors, whereas the mRNAs from the control rat and dog tumors failed to hybridize. In general, the hybridization patterns observed in the mRNAs from the animal tumors were simpler than the corresponding patterns observed in the human tumor-derived mRNAs, with a predominant and often single broad band being identified in the region of 1.5–1.6 kb. Lower abundance transcripts of approximately 3.2, 4.2, and 4.8 kb were observed in the mRNA from the dog lymphosarcoma (LSA^+ in Fig. 3). Based on their translatability in vitro, each of these mRNA preparations appeared to be of high quality.

The sequence similarity between human PTH and the human PTH-like peptide in the proximal amino terminus presumably accounts for the PTH-like effects of the tumor-derived peptide. To extend the findings from Northern blotting and further focus on this region in the animal-tumor mRNAs, a hybridization-arrest experiment was performed using a complementary oligonucleotide spanning the region of sequence homology (Fig. 4). As shown in Table I, medium from Xenopus oocytes injected with each of the animal tumor mRNAs reacted strongly in the cytochemical bioassay for PTH, and in each case preincubation with the oligonucleotide completely inhibited this mRNA-dependent activity. To rule out nonspecific inhibition in the hybridization-arrest experiment, mRNA (1 μg/μl) from line SKRC-1 was injected alone and following preincubation with an identical concentration (10 pmol/μl) of both the sense and the complementary oligonucleotide (resulting in bioassay values of 72.2, 78.8, and 0.6 pgeq/ml, respectively).

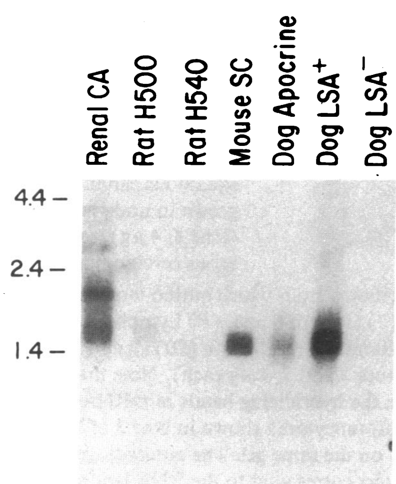

Figure 3. Northern blot analysis of mRNAs prepared from animal tumors. Each lane contained 4 μg poly $(A)^+$ RNA. The renal carcinoma is line SKRC-1, and LSA+ and LSA− correspond to lymphosarcomas associated and unassociated with HHM. The autoradiogram is a 12-h exposure.

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                    -1 +1                                    10
             PTH:   Lys Arg Ser Val Ser Glu Ile Gln Leu Met His Asn Leu Gly Lys
PTH-like Peptide:   Lys Arg Ala Val Ser Glu His Gln Leu Leu His Asp Lys Gly Lys
            cDNA: 5' AAA AGA GCT GTG TCT GAA CAT CAG CTC CTC CAT GAC AAG GGG AAG 3'
 Oligonucleotide: 3' TTT TCT CGA CAC AGA CTT GTA GTC GAG GAG GTA CTG TTC CCC TTC 5'
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Figure 4. Oligonucleotide used in the hybridization-arrest experiment. The figure depicts the region of homology between human PTH and the human PTH-like peptide, the cDNA sequence of the PTH-like peptide in this region, and the complementary 45-base oligonucleotide synthesized. The amino-acid sequence is numbered − or + relative to the initial amino acid of the mature proteins, and shared amino acids are underlined. The sense oligonucleotide used as a negative control with mRNA from line SKRC-1 corresponds to the cDNA sequence.

Table I. Hybridization-Arrest Experiment Using Animal Tumor-derived mRNAs

Tumor	Without oligonucleotide	With oligonucleotide
	pgeq/ml	*pgeq/ml*
Rat H500	80.3	0.8
Mouse	42.9	0.6
Dog apocrine	34.0	1.0
Dog lymphosarcoma	58.5	0.7

Cytochemical bioassay results in media from Xenopus oocytes injected with mRNAs (1 μg/ul) with or without preincubation with a 45-base complementary oligonucleotide (10 pmol/μl) spanning the sequence of homology between human PTH and the human PTH-like peptide. Media from oocytes injected with mRNA (1 μg/ul) prepared from the control dog lymphosarcoma (LSA^- in Fig. 3) failed to stimulate the cytochemical assay (1.1 pgeq/ml); mRNA from the rat H540 tumor was not injected.

Discussion

Using a probe corresponding to the coding region of the human tumor-derived, PTH-like peptide, we examined by Northern analysis mRNAs prepared from a variety of human and animal tumors associated or unassociated with HHM. All mRNAs from HHM-associated tumors contained one or more hybridizing transcripts, whereas mRNAs prepared from histologically similar tumors unassociated with HHM failed to hybridize with the probe. The human HHM-associated tumors included several examples of nonprototypical tumors (the breast carcinomas) and several examples of tumors which appeared to be clinically benign (the pheochromocytomas). Each human tumor-derived mRNA displayed multiple hybridizing transcripts, numbering up to five, with a highly reproducible pattern being observed in mRNAs from multiple examples of a single cell type (renal carcinoma) and with a similar pattern being observed in mRNAs from apparently benign tumors (the pheochromocytomas).

Southern blot analysis of genomic DNA from multiple species as well as chromosome mapping suggests that the gene for the PTH-like peptide is a single-copy gene (11). It would follow that the most likely explanation for the multiple transcripts observed on Northern analysis is alternative RNA processing (29). Additional evidence favoring this mechanism includes the identification of a second cDNA clone that encodes a longer peptide with a different C-terminus.[4] This putative mechanism is not confined to transformed cells, in that multiple transcripts have been observed in mRNAs prepared from benign tumors (Fig. 3) and from normal human keratinocytes grown in primary culture (11). These findings suggest that the gene for the PTH-like peptide may have a complex organization and also raise the possibility that several PTH-like peptides may be produced by one or another tumor or tissue. Direct data bearing on these questions are presently unavailable.

The animal HHM-associated tumors examined included three spontaneous tumors from the rat and dog (12–14) and a carcinogen-induced squamous carcinoma model in the mouse (15). Several of these tumors have been extensively studied (12, 13), but peptide sequence data have yet to be published.[5] Poly $(A)^+$ RNA prepared from each of these tumors contained mRNA-dependent PTH-like activity when injected into Xenopus oocytes and assayed in a sensitive bioassay and displayed a predominant hybridizing band of ~ 1.5–1.6 kb on Northern analysis. The results of the hybridization-arrest experiment complement those of the Northern analysis and indicate that the PTH-like sequence in the extreme amino terminus of the tumor-derived peptides is highly conserved. These findings cannot be taken as evidence of sequence identity, since the hybridization-arrest technique allows for a degree of mismatching of base pairs (26).

Amino-terminal peptides 34 to 36 amino acids in length have recently been synthesized by three groups and shown to be active in PTH-sensitive bioassays in vitro and to induce marked hypercalcemia when infused into rats (30–32).

In summary, our findings corroborate previous data implicating the production of one or more PTH-like peptides by a variety of tumors associated with HHM and indicate a high degree of conservation of the PTH-like sequence in the peptide(s) produced by mammalian tumors.

Acknowledgments

We thank A. Caplan for technical assistance, N. Canetti and P. Strumpf for preparation of the manuscript, and C. Tschudi and E. Ullu for helpful advice.

Supported by National Institutes of Health grant AR-30102 (A. E. Broadus), grants from the Veterans Administration (K. L. Insogna and A. F. Stewart), and a James Hudson Brown-Alexander B. Coxe Fellowship from the Yale School of Medicine (K. Ikeda).

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