Parathyroid Hormone-Related Protein Induces Cachectic Syndromes without Directly Modulating the Expression of Hypothalamic Feeding-Regulating Peptides

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Abstract

Purpose: Parathyroid hormone-related protein (PTHrP) is a causative factor of humoral hypercalcemia of malignancy (HHM) and concurrent anorexia and wasting. Because changes in the expression of hypothalamic feeding-regulating peptides can directly affect appetites and thereby can cause anorexia and wasting, we addressed whether the cachectic syndromes induced by PTHrP rely on the action of hypothalamic feeding-regulating peptides.

Experimental Design: Rats were inoculated with a LC-6 human cancer xenograft that secreted PTHrP, and the mRNA levels of the hypothalamic feeding-regulating peptide genes and serum leptin levels were examined before and after the development of HHM. Plasma leptin levels were also reduced in cachectic rats, and the administration of anti-PTHrP antibody to the cachectic rats not only improved the cachectic symptoms but also restored the mRNA levels of these orexigenic and anorexigenic peptides, except for orexin. Thus, PTHrP induces HHM and concurrent cachectic syndromes by mechanisms other than directly modulating the leptin or hypothalamic feeding-regulated peptides.

The severity of cachexia from such illnesses as cancer, end-stage renal disease, and HIV may be the primary determining factor in both the quality of life and the eventual mortality (1, 2). Anorexia-cachexia syndrome is observed in a large number of patients in the advanced stages of cancer (3, 4). It is a complex disease characterized by several metabolic and behavioral abnormalities, including early satiety, weakness, general fatigue, depression, and weight loss. Although the etiology of the cachexia is not well defined, several hypotheses have been explored for cytokines, hormones, neuropeptides, neurotransmitters, tumor-derived factors, and others (1, 5–8). Previous studies led to the hypotheses that body weight is regulated by a feedback loop in which peripheral signals report nutritional information to an integrating center in the hypothalamus of the brain and that neuropeptides are essential molecules within the hypothalamus (9–13).

It is known that appetite is under chemical control and that feeding behavior is regulated by various hypothalamic peptides, orexigenic peptides, such as agouti-related protein (AgRP), neuropeptide Y (NPY), and orexins (hypocretins), and anorexigenic peptides, such as cocaine- and amphetamine-regulated transcript (CART), proopiomelanocortin (POMC), corticotropin-releasing factor (CRF), α-melanocyte-stimulating hormone, and leptin (14, 15). Among these peptides, functions of leptin have been well studied in mice and rats lacking the functional leptin or its receptor. Leptin is the product of the ob gene, and fasting suppresses ob gene expression in adipocytes (16, 17), leading to a decrease in the plasma concentration of leptin (18). Leptin treatment impairs fasting-induced neuroendocrine responses in the gonadal-adrenal-thyroid axis (19), and leptin deficiency due to mutation of the ob gene (ob/ob mouse) and leptin resistance due to mutation of the leptin receptor (db/db mouse and fa/fa rat) cause severe obesity (16, 20, 21). Furthermore, NPY mRNA levels in the arcuate nucleus (Arc) of ob/ob mouse, db/db mouse, and fa/fa rat were significantly increased in comparison with controls (22, 23).

Previously, we showed that animals carrying tumors secreting parathyroid hormone-related protein (PTHrP) exhibited...
humoral hypercalcemia of malignancy (HHM), reduced food intake, and body weight loss, and administration of the humanized anti-PTHrP antibody raised against the NH2-terminal 34 amino acids of the human PTHrP (PTHrP1-34) improved these symptoms (24, 25). Because the anti-PTHrP antibody augmented the food intake in cachectic animals, we speculated that PTHrP secreted from tumor induced HHM and concurrent anorexia by affecting the gene expression of hypothalamic feeding-regulating peptides and leptin.

In the present study, we examined whether orexigenic and anorexigenic peptides are involved in the PTHrP-induced anorexia and cachexia and found that PTHrP induced anorexia and cachexia without directly up-regulating the anorexigenic peptides or down-regulating the orexogenic peptides in the hypothalamus.

Materials and Methods

Cells and animals. Human lung cancer cell line LC-6-ICJ originating from human large cell lung cancer was purchased from the Central Institute for Experimental Animals (Kawasaki, Japan). The cells were maintained in vitro in nude mice (BALB/cAnNCrj-nu/nu), and small pieces of tumor tissues (−10 mm3) were s.c. implanted into 5-week-old male F344/N Iel-nu nude rats. The rats displayed hypercalcemia and weight loss between 42 and 60 days after implantation of the tumor, and those with blood ionized calcium (iCa) levels higher than 1.8 mmol/L and at least 0.5 mmol/L higher than the nontumor-bearing rats were used as HHM rats (24, 25–27). Day 0 represents the day when the tumors were implanted. Nude mice and nude rats purchased from Charles River Japan, Inc. (Yokohama, Japan) and Clea Japan, Inc. (Tokyo, Japan), respectively, were kept in sterilized cages.

Drugs. The humanized anti-PTHrP antibody raised against the NH2-terminal 34 amino acids of the human PTHrP (PTHrP1-34, Ref. 25) was dissolved in saline. Rats were given 3 mg/kg of the anti-PTHrP antibody i.v. once weekly.

Experimental procedures. The body weight and iCa in nontumor-bearing rats and HHM rats were measured 28, 38, 46, 52, 56, 60, and 63 days after implantation of the tumor. Blood was collected from the tail vein, and the concentration of iCa was measured by the electrode method using a Ca2+/pH electrolyte analyzer (Bayer 634, Bayer). The blood samples were collected at 3-day intervals, and the concentration of iCa was measured by the electrode method using a Ca2+/pH electrolyte analyzer (Bayer 634, Bayer Diagnostics, Sudbury, United Kingdom).

In situ hybridization histochemistry. In situ hybridization histochemistry was done on frozen 12-μm-thick coronal brain sections cut on a cryostat at −20°C, thawed, and mounted onto gelatin/chrome alun-coated slides. Brain tissue was stored at −80°C before cutting. The locations of the paraventricular nucleus (PVN), Arc, and lateral hypothalamic area (LH) were determined according to coordinates given by the atlas of Paxinos and Watson (28). Ten sets of two sections containing the PVN and four sections containing the Arc and LH were used from each rat to measure the density of autoradiography. In situ hybridization procedures have been described in detail previously (29). In brief, hybridization was done at 37°C overnight in a 45 μL buffer solution consisting of 50% formamide and 4× SSC (1× SSC = 150 mmol/L NaCl, 15 mmol/L sodium citrate), which contained 500 μg/mL sheared salmon sperm DNA (Sigma, St. Louis, MO), 250 μg/mL baker’s yeast total RNA (Roche Molecular Biochemicals GmbH, Mannheim, Germany), 1× Denhardt’s solution, and 10% dextran sulfate with molecular weight 500,000 (Sigma). The hybridization was done under a Nescofilm coverslip (Bando Chemical IMD Ltd., Osaka, Japan). [35S]3’-end-labeled deoxyoligonucleotides complementary to transcripts coding for AgRP (5’-CGACCCGGAGAGACGTCCG- GGTCTGAGATGCTAACCTCTGGC-3’; NPY (5’-GAGTAGATCCT- GCGCATGTCTGCTGGGCGGC-3’; CART (5’-TGGGCCGTTATCTTCTTCTCATAGATCGGAATGC-3’; POMC (5’- CCTCTGCCCAGCCGGTGGCCCAGCAAGTGGTTCATGAGC-3’), CRF (5’-CAGTTTCCTGTGTGTGAGCTGTGCTGCTGAC- AVRAGTTCCCTGCTCCCTGGC-3’), thyrotropin-releasing hormone (TRH; 5’-CTTTTTTCTCTCTCTCTCAATTGCTGGCTTTG- GAT-3’), orexin (5’-TCCGGACGGCGGAGAGACGTCCG- TACTGCTCTGCTCCCTGGC-3’), and melanin-concentrating hormone (MCH; 5’-CCAGCGGGTGGTAGACTGCTCCCAGCAT-3’).
The specificity of the probes has been described previously, except for CART and MCH (23, 29–32). The specificity of the probes for CART and MCH was established from the nucleotide database at the National Center for Biotechnology Information. Total counts of $6 \times 10^5$ cpm/slide for AgRP, NPY, CRF, POMC, CART, and TRH and $4 \times 10^5$ cpm/slide for orexin and MCH transcripts were used. Hybridized sections containing the PVN, the Arc, and the LH were exposed to autoradiography film (Hyperfilm, Amersham, Buckinghamshire, United Kingdom) for 4 days for orexin and 7 days for AgRP, NPY, CRF, POMC, CART, TRH, and MCH. The autoradiographic images were quantified using an MCID imaging analyzer (Imaging Research, Inc., St. Catherines, Ontario, Canada). The images were captured by a charge-coupled device camera (Dage-MTI, Inc., Michigan City, IN) at $\times 40$ magnification. The mean absorbance of the autoradiographs was measured by comparing with simultaneously exposed $^{14}$C microscale samples (Amersham). $^{14}$C was used as the standard for quantification of the absorbance of autoradiographs for in situ hybridization histochemistry. The standard curve was fitted by the absorbance of the $^{14}$C microscale on the same film.

**Statistical analysis.** All data are given as mean ± SE calculated from the results of the in situ hybridization histochemistry. Each group within an experiment was compared with the control group. The data were analyzed using a one-way fractional ANOVA followed by a Bonferroni correction for multiple comparisons. The changes in body weight, iCa, and plasma leptin were also statistically analyzed using one-way ANOVA followed by a Bonferroni correction for multiple comparisons. Statistical significance was defined as $P < 0.05$.


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**Results**

Expression of hypothalamic peptides, orexigenic peptides, and anorexigenic peptide mRNAs in nontumor-bearing rats, HHM rats, and rats given with anti-PTHrP antibody. Consistent with previous results (24, 26, 27), body weight of HHM rats significantly decreased in comparison with nontumor-bearing rats after day 30 (30 days after tumor implantation) but significantly increased after HHM rats were given the anti-PTHrP antibody (Fig. 1A). In addition, the level of iCa in HHM rats became significantly higher than that of nontumor-bearing rats after day 30 but it was decreased after HHM rats received the anti-PTHrP antibody (Fig. 1B). Single administration of 3 mg/kg anti-PTHrP antibody to HHM rats restored blood calcium for at least 2 weeks. In addition, these rats continued to gain weight and showed increased food uptake for at least 2 weeks compared with vehicle control (26). Furthermore, repeated weekly administration of the anti-PTHrP antibody nearly completely restored blood calcium and body weight for at least 6 weeks (25).

To address whether feeding-regulated neuropeptides are involved in the wasting in this animal model, we first examined the expression of the orexigenic peptide genes in HHM rats and compared it with that in the control nontumor-bearing rats at the time body weights of the rats bearing LC-6 began to decrease (day 53) and after they reached a plateau (day 63). The mRNA levels of the genes for orexigenic peptides, AgRP and NPY, in the Arc were significantly higher in HHM rats than in
Fig. 3. Expression of mRNA for CART (A, B, C, and G) and POMC (D, E, F, and H) in the Arc of the nontumor-bearing rats (Normal), HHM rats (HHM), and rats injected with anti-PTHrP antibody (HHM + Ab) 53 and 63 d after implantation of tumor. Representative autoradiographs of sections hybridized to a 35S-labeled oligodeoxynucleotide probe complementary to mRNA for CART (A–C) and POMC (D–F) in the Arc 63 d after transplantation of tumor showing signal intensity from high (white) to low (black). A and D, sections from a nontumor-bearing rat. B and E, sections from a HHM rat. C and F, sections from a HHM rat given with anti-PTHrP antibody. Bar, 1 mm. Columns, mean (n = 6); bars, SE. **, P < 0.01, compared with nontumor-bearing rats; ##, P < 0.01, compared with rats given with anti-PTHrP antibody.

Fig. 4. Expression of mRNA for CRF (A, B, C, and G) and TRH (D, E, F, and H) in the PVN of nontumor-bearing rats (Normal), HHM rats (HHM), and rats injected with anti-PTHrP antibody (HHM + Ab) 53 and 63 d after implantation of tumor. Representative autoradiographs of sections hybridized to a 35S-labeled oligodeoxynucleotide probe complementary to mRNA for CRF (A–C) and TRH (D–F) in the PVN 63 d after transplantation of tumor showing signal intensity from high (white) to low (black). A and D, sections from a nontumor-bearing rat. B and E, sections from a HHM rat. C and F, sections from a HHM rat given with anti-PTHrP antibody. Bar, 1 mm. Columns, mean (n = 6); bars, SE. **, P < 0.01, compared with nontumor-bearing rats; ##, P < 0.01, compared with rats given with anti-PTHrP antibody.
nontumor-bearing rats on days 53 and 63 (Fig. 2). Administration of the anti-PTHrP antibody to HHM rats showed reduced levels of the AgRP and NPY mRNA compared with levels in the control rats (Fig. 2). Next, we examined the expression of the anorexigenic genes in HHM rats. The mRNA levels of the genes for the anorexigenic peptides, CART and POMC in the Arc and CRF in the PVN, in HHM rats were all lower than those in nontumor-bearing rats on days 53 and 63 (Figs. 3 and 4A, B, C, and G). Administration of the anti-PTHrP antibody to the HHM rats significantly increased the levels of CART, POMC, and CRF mRNA, but their levels were still lower than those in the nontumor-bearing rats even after HHM rats received the anti-PTHrP antibody (Figs. 3 and 4A, B, C, and G). We also examined the expression of the TRH gene in the PVN and found that the level of TRH mRNA was more or less the same between HHM and nontumor-bearing rats (Fig. 4D, E, F, and H), and the administration of the anti-PTHrP antibody had no effect.

The mRNA level of the orexin gene in the LH of HHM rats was similar to that of the nontumor-bearing rats on day 53 but became lower than nontumor-bearing rats by day 63 and was not increased by the administration of the anti-PTHrP antibody (Fig. 5). There was no significant difference in the mRNA level of the MCH gene in the LH of HHM rats given the anti-PTHrP antibody and nontumor-bearing rats (Fig. 5).

Expression of the NPY gene has been reported to be affected by leptin (33). Therefore, we also examined the plasma leptin levels in HHM rats. Concentration in plasma of leptin in HHM rats was significantly lower than that in nontumor-bearing rats on days 53 and 63 (Fig. 6). Administration of the anti-PTHrP antibody to HHM rats greatly increased the plasma leptin level, but it was still lower than that of the nontumor-bearing rats.

**Discussion**

PTHrP has been isolated as a principal factor responsible for HHM (34). As previous studies have shown, rats carrying the LC-6 xenograft display HHM and concurrent anorexia, and administration of anti-PTHrP antibody restored blood calcium, food intake, and body weight in these rats (24, 26, 27). Capparelli et al. (35) reported that s.c. administration of osteoprotegerin improved hypercalcemia in mice bearing the Colon-26 tumor, which produced and secreted PTHrP into plasma and induced hypercalcemia; however, body weight loss and high levels of PTHrP in plasma remained. The results suggest that the anorexia and wasting in these mice did not solely depend on hypercalcemia and that tumor-secreted PTHrP might be directly involved.

In the present study, we focused on the hypothalamic function in HHM rats treated or untreated with anti-PTHrP antibody because the hypothalamus is well known to be an integrative site in the regulation of feeding (36). The mRNA levels of orexigenic peptides, such as AgRP and NPY, in HHM rats were significantly higher than those seen in nontumor-bearing rats, whereas the mRNA levels of anorexigenic peptides, such as CART, POMC, and CRF, in HHM rats were lower than those seen in nontumor-bearing rats. Administration of anti-PTHrP antibody to HHM rats restored these mRNA levels. The
results suggest that the HHM rats were eager to eat but, in fact, could not, and restoration by the administration of anti-PTHrP antibody was the consequence of the improvement of their feeding. Although it has been generally believed that anorexia is accompanied by the up-regulation of orexigenic peptides and/or down-regulation of orexigenic peptides, the hypothalamic signals clearly tended to stimulate feeding in HHM rats. Elevated plasma levels of ghrelin have also been reported in the G361 human melanoma xenograft model (37) and in lung cancer patients who developed cachexia and chemotherapy-induced anorexia (38).

In the present study, the plasma leptin level also declined in the HHM rats and anti-PTHrP antibody restored it. Many appetite-regulating peptides are under the control of leptin (14, 15), and leptin activates neuroendocrine cells in the hypothalamus, particularly in the Arc, which contains both orexigenic and anorexigenic peptides, such as AgRP, NPY, CART, and POMC (33, 39, 40). Therefore, it seems that the adipose tissues and hypothalamus where leptin is produced and acts, respectively, are not the primary target sites of PTHrP to induce anorexia and cachexia. On the other hand, the mRNA levels of orexins and MCH, which are expressed in the neurons of the LH and enhance feeding (41, 42), were not markedly altered in HHM rats, and anti-PTHrP antibody had little effect. Thus, PTHrP may affect factors and mechanisms that function between hypothalamic feeding-regulating peptides and central nervous system pathways downstream of these peptides. However, we cannot rule out the possibility that PTHrP directly activates neurons in the hippocampus because PTHrP induced a marked elevation in the concentration of cytosolic free calcium in a hippocampal single neuron (43) and the calcium-sensing receptor is localized in the brain, especially in the subfornical organ, olfactory bulbs, and hippocampus in mice and rats (44, 45).

In conclusion, we showed that increased plasma levels of PTHrP cause HHM and concurrent anorexia and cachexia without up-regulating plasma leptin levels and anorexigenic peptide expression and down-regulating orexigenic peptide expression. Thus, modulation of leptin or the hypothalamic feeding-regulating peptides is not the primary action of PTHrP that induces anorexia and cachexia, and other mechanisms are involved in the PTHrP-induced anorexia and cachexia.

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References


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