Hypothalamic Appetite-Regulating Neuropeptide mRNA Levels in Cachectic Nude Mice Bearing Human Tumor Cells

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We previously reported that the human melanoma cell line, SEKI, induces severe weight loss in nude mice. In the present study, we examined the expression of weight-regulating neuropeptide mRNAs in the hypothalamus of this cancer cachectic model by using a sensitive quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) method and in situ hybridization. mRNA levels of neuropeptide Y (NPY) and corticotropin-releasing hormone (CRH) in the whole hypothalamus were elevated significantly in the SEKI mice as compared with control mice. In situ hybridization showed that NPY and CRH mRNA were upregulated in the arcuate nucleus and the paraventricular nucleus, respectively. There were no significant differences in melanin-concentrating hormone (MCH), orexin (OX), and cholecystokinin mRNA levels between the SEKI and control mice. These results suggest that the NPYergic system is functioning in the rodent model of cancer cachexia; however, the role of the CRHergic system in energy homeostasis remains to be elucidated. This is the first report of the hypothalamic neuropeptide response to cachexia-inducing human cells.

BODY WEIGHT LOSS characterized by depletion of adipose tissue and skeletal muscle mass is a principal sign of cancer cachexia, a syndrome frequently observed in patients at the terminal stage of cancer.[1] Although the pathogenesis of cancer cachexia is apparently multifactorial and different among cancer patients, an alteration in the regulatory mechanism that normally maintains a constant lean body mass is likely to contribute to the progressive body weight loss.

The stability of lean body mass is normally ensured by the regulation of appetite and energy expenditure, in which a variety of central neural pathways are involved including hypothalamic neurons containing neuropeptide Y (NPY), melanin-concentrating hormone (MCH), orexin (OX), corticotropin-releasing hormone (CRH), and cholecystokinin (CCK).[2] Previous studies showed that the hypothalamic concentration of NPY, release of NPY into hypothalamic interstitial fluid, and feeding response following intrahypothalamic injection of NPY were all decreased in anorectic tumor-bearing rats when compared with nontumor-bearing counterparts.[3-5] In contrast, the hypothalamic NPY mRNA content was markedly increased in tumor-bearing rats.[6,7] Thus, the NPYergic pathway has been implicated as one of the hypothalamic neural pathways whose activity is altered in cancer cachexia, although its exact role in the development of tumor-induced body weight loss remains to be elucidated. The involvement of other hypothalamic signaling systems in cancer cachexia is even less well understood.

We previously demonstrated that some human tumor cells, including SEKI melanoma cells, induce body weight loss in tumor-bearing nude mice.[8,9] SEKI cells produce a large amount of leukemia inhibitory factor (LIF), which causes an inhibition of lipoprotein lipase activity and may directly act on the adipose tissue.[8,9] In the present study, we examined the status of the central neural system regulating energy homeostasis by examining hypothalamic levels of mRNAs for regulatory peptides in this experimental model. We used a sensitive competitive reverse transcription (RT)-polymerase chain reaction (PCR) method, which permitted quantitative evaluation of multiple mRNA species in a single hypothalamic specimen of nude mice. Furthermore, the elevated expressions of NPY and CRH mRNAs were localized to the specific nuclei by use of in situ hybridization.

MATERIALS AND METHODS

Animals

Five-week-old female BALB/C-*nu*/*nu* mice were purchased from Charles River Japan (Atsugi, Japan) and adapted to laboratory conditions for 1 week. These mice were housed individually in plastic cages on hard wood chips, in a temperature-controlled chamber under 12:12-hour light-dark cycles, with light on at 7:00 AM. All mice were maintained ad libitum on tap water and a breeding diet (Oriental Yeast, Tokyo, Japan) placed on the ground.

Experimental Groups and Tumor Inoculation

Six mice were euthanized by decapitation after 1 week of environmental adaptation and were used as controls of day 1. The other 68 animals were randomly allocated into 4 groups each consisting of 17 mice. Mice of 1 group (SEKI group) were subcutaneously inoculated in both sides of the flank region with approximately 50 mg of SEKI human melanoma cells that was taken from a donor of the animal tumor colony as described previously.[9] Mice of another group (A375 group) were similarly inoculated with A375 human melanoma cells. Another 17 mice (Sham group) were subjected to sham tumor inoculations using an empty trocar. The remaining 17 mice (pair-fed group) were matched in the amount of food intake to the SEKI mice and used as a matched pair feeding control. The food intake was matched by allowing only enough chow to maintain individual food intake equal to that of the matched SEKI group mice. All inoculation procedures were performed under ether anesthesia.

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Table 1. Nucleotide Sequences of Primers Used in the Preparation of Internal Standard, Reverse Transcription, and PCR Analysis for Neuropeptides and β-Actin mRNA

Nucleotide Sequence	Position
NPY*	
1 5′-GACATGGCCAGATACTACTCCGCT-3′	46-69
2 5′-GGGATGAGATGAGATGAGGGTGGA-3′	248-271
3 5′-TGGTTTCAGGGGATG-3′	266-280
4 5′-GACATGGCCAGATACTACTCCGCTCACTACATCAATCTCATCACCAG-3′	46-98
MCH*	
1 5′-AACTCACGGGCTGCCACTGAGTC-3′	276-298
2 5′-GGTAGACTCTTCCCAGCATACACC-3′	455-478
3 5′-CCAACATGGTCGGTA-3′	475-489
4 5′-AACTCACGGGCTGCCACTGAGTCTAAAACCTTACCTTGCTCTGAAAGG-3′	276-329
OX	
1 5′-CCTGAGCTCCAGGCACCATGAACT-3′	−17-7
2 5′-TGGTTACCGTTGGCCTGAAGGAGG-3′	243-266
3 5′-GTCAGGATGCCAGCTGCGTGGTTA-3′	261-284
4 5′-CCTGAGCTCCAGGCACCATGAACTTTACAAAGGTTCCCTGGGCCGCCG-3′	−7-37
CRH*	
1 5′-AGGCATCCTGAGAGAAGTCCCTCT-3′	433-458
2 5′-TCTTCACCCATGCGGATCAGAACC-3′	1358-1381
3 5′-CAGCGGAGGAAGTATTCTTCACCC-3′	1373-1396
4 5′-AGGCATCCTGAGAGAAGTCCCTCTGCAGCAGTGCGGGCTCACCTACC-3′	433-485
CCK	
1 5′-GGTAGTCCCTGCAGAAGCTACGG-3′	1198-1220
2 5′-ATCCAGCCCATGTAGTCCCGGTC-3′	5497-5520
3 5′-CGATGGGTATTCGTAGTCCTCGG-3′	5540-5562
4 5′-GGTAGTCCCTGCAGAAGCTACGGAGGAGCAGCGGGCGGAAGAGGCGCC-3′	1198-1252
β-actin	
1 5′-GGTATGGAATCCTGTGGCATCCAT-3′	724-747
2 5′-TGATCCACATCTGCTGGAAGGTGG-3′	971-994
3 5′-ACTCATCGTACTCCTGCTTGCTGA-3′	992-1015
4 5′-GGTATGGAATCCTGTGGCATCCATGAATTCAATTCCATCATGAAGTGT-3′	724-777

NOTE. Nucleotide positions are based on the published reference for NPY,[10] MCH,[11] OX,[12] CRH,[13] CCK,[14] and β-actin.[15] 1, sense primers for PCR; 2, antisense primers for PCR; 3, primers for reverse transcription; 4, sense primers for preparation of internal standard.

*Because there was no report about the nucleotide sequence of mouse NPY, MCH, and CRH cDNA, those nucleotide sequences were determined by RT-PCR using primers designed on the basis of rat nucleotide sequences reported previously.

The body weight of the mice and the length, width, and height of the tumors were measured every other day. Food intake was measured daily and determined by subtracting the amount of uneaten mice chow from the initial amount. Mice from each group were euthanized by decapitation between 3:00 to 4:30 PM on day 9 (n = 6) and day 19 (n = 11). After decapitation, blood was collected from the trunk into a plastic plate containing heparin, and the hypothalamus was removed immediately, snap-frozen in liquid nitrogen, and stored at −80°C until mRNA extraction. Nontumor body weight was calculated by subtracting the tumor weight, which was estimated by using the formula, $\pi abc/6$ (mm^3), from the whole body weight.

Preparation of Internal Standard RNA

Portions of the mouse NPY, MCH, OX, CRH, CCK, and β-actin cDNA were amplified by PCR with primers as described in Table 1.[10-15] cDNAs with a 6-bp deletion were generated by PCR using synthetic primers containing an internal 6-bp deletion. The cDNA fragments containing the deletion were ligated into the pCR II-TOPO vector (Invitrogen, Carlsbad, CA) in the direction that allowed in vitro transcription of the sense strand RNA for the neuropeptides from the T7 promoter. RNA was synthesized with an in vitro RNA transcription kit (Stratagene, La Jolla, CA). The synthesized RNA was purified through a QIAGEN tip 20 (Qiagen, Valencia, CA). Denaturing agarose gel electrophoresis of the RNA products revealed discrete bands of the correct size, verifying the integrity of RNA. The synthetic RNA was diluted in a buffer (10 mmol/L Tris, 1 mmol/L EDTA, pH 8.0) containing 100 mg/mL yeast tRNA.

Quantitative Analysis of Neuropeptide mRNA by RT-PCR

Total RNA fractions were isolated from the hypothalamic block by using RNeasy total RNA kit (Qiagen). cDNAs for NPY, MCH, OX, CRH, CCK, and β-actin mRNA were generated in a single tube by reverse transcription of total RNA (1 μg) in the presence of 0.1 to 100 attomoles of the internal standard RNAs, with a mixture of antisense primers specific to each mRNA (Table 1). The cDNAs for each mRNA were amplified separately by PCR with specific primers as shown in Table 1, the sense primer of which was labeled with fluorescein. PCR conditions were: 94°C for 1 minute followed by 20 cycles (NPY, MCH, OX, and β-actin), 24 cycles (CCK) or 27 cycles (CRH) at 94°C for 1 minute; 60°C (CRH and CCK) or 62°C (NPY, MCH, OX, and β-actin) for 1 minute: 72°C for 2 minutes, and a final extension at 72°C for 5 minutes. In these conditions, the amounts of PCR products were almost proportional to the amount of cDNA templates. The PCR products were analyzed with a capillary genetic analyzer (ABI PRISM 310, Perkin-Elmer, Foster City, CA), and the fluorescent peak areas for the PCR product from the sample mRNA and the internal standard RNA were measured. The amounts of mRNA was determined by multiplying the amount of the internal standard RNA by the ratio of the fluorescent

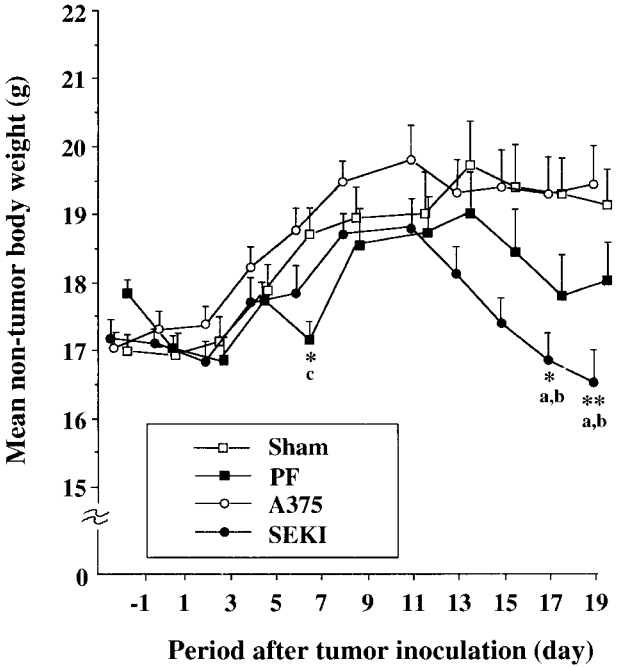

Fig 1. Mean (+SE) nontumor body weight of Sham, PF, A375, and SEKI mice after tumor inoculation. Nontumor body weight was calculated by subtracting the tumor weight from the whole body weight. *$P < .05$ and **$P < .01$. (A) Sham *v* SEKI, (B) A375 *v* SEKI, (C) A375 *v* PF.

peak area of the PCR product from the specific mRNA to that derived from the internal standard RNA. Neuropeptide mRNA values were normalized with β-actin mRNA values.

NPY and CRH In Situ Hybridization

Four SEKI tumor transplanted mice were killed by decapitation when their nontumor weight decreased to less than 85% of Sham mice and their brains were collected for in situ hybridization. Four Sham-operated mice served as controls.

For the NPY cDNA probe, a synthetic antisense oligonucleotide (5′-CTCTGCTGGC GCGTCCTCGC CCGGATTGTC CGGCTTG-GAG GGGTA-3′) was designed to be complementary to nucleotide residues 171-215 of rat NPY cDNA,[16] which was highly homologous to the corresponding human sequence (42 of 45 nucleotides are identical). For the CRH cDNA probe, a synthetic antisense oligonucleotide (5′-ATAATCTCCA TCAGTTTCCT GTTGCTGTGA GCTTGCTG-3′), 38 nucleotides in length, was designed to be complementary to nucleotide residues 1715-1752 of rat CRH (Gen Bank, accession no M54987), which was identical to the corresponding pig sequence. These oligonucleotides were labeled with ^{35}S-deoxyadenosine triphosphate (dATP), using terminal deoxynucleotidyl transferase (Promega, Madison, WI). Cryostat sections, 14 mm in thickness, were prepared from the frozen mouse brain and mounted on glass slides precoated with 3-aminopropyltriethoxysilane. They were fixed with 4% paraformaldehyde for 10 minutes and acetylated for 10 minutes with 0.25% acetic anhydride in 0.1 mol/L triethanolamine-hydrochloride. After prehybridization, the sections were incubated in a hybridization buffer containing 10,000 cpm/mL of ^{35}S-labeled probes. The methodology for hybridization has been described elsewhere.[17] The sections were dipped in Kodak NTB2 nuclear track emulsion and exposed for 1 month. The autoradiograms were quantified using NIH image Version 1.61 software (National Institutes of Health, Bethesda, MD).

Measurement of Plasma Corticosterone and LIF

Plasma corticosterone levels were measured with a radioimmunoassay kit (Amersham Life Science, Arlington Heights, IL) and plasma LIF was determined with an enzyme-linked immunosorbent assay kit for human LIF (R & D Systems, Minneapolis, MN) using recombinant mouse LIF (R & D Systems) as a standard.

Statistical Evaluation

All data are expressed as means and SE. The statistical significance of difference in food intake and body weight was analyzed using repeated measure analysis of variance (ANOVA) and 1-way ANOVA with post hoc test using the Scheffé test. The data for mRNA levels, as well as for plasma LIF and corticosterone levels, were compared between groups using 1-way ANOVA coupled to a post hoc Scheffé test. A P value of $< .05$ was taken as significant.

RESULTS

Nontumor body weight gain was similar in all groups for the first 11 days after tumor implantation. Thereafter, body weight of the SEKI mice declined continuously as the tumor grew, while the body weights of the control groups remained stable (Fig 1). Analysis of simple group effect on each time point revealed that nontumor body weight was significantly lower in SEKI than in Sham or A375 mice on days 17 and 19, although repeated measures ANOVA showed no significant difference among groups. Final nontumor body weights were 16.6 $\pm$ 0.5 g for SEKI, 19.5 $\pm$ 0.6 g for A375, 19.2 $\pm$ 0.5 g for Sham, and 18.1 $\pm$ 0.6 g for PF. Mean tumor weight in tumor-bearing mice euthanized on day 19 was 2.5 $\pm$ 0.5 g for SEKI and 1.3 $\pm$ 0.2 g for A375 (Fig 2), which represented 12.9% and 6.4% of total body weight, respectively. Cumulative food intake was not significantly different among groups (Fig 3). Repeated measures and 1-way ANOVA revealed that the group effect was not significant at any time point during the entire experiment.

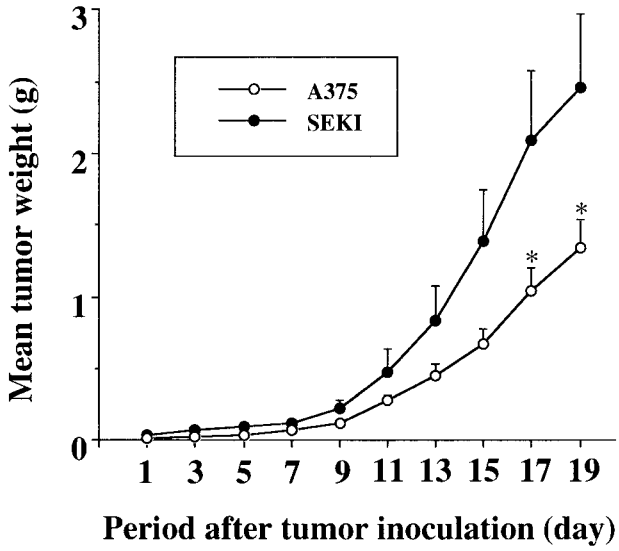

Fig 2. Mean (+SE) estimated and actual (days 9 and 19) tumor weight of A375 and SEKI mice. Mean tumor weight was estimated from cubic measurements except the value on days 9 and 19 for which the actual weights of resected tumors were measured. *$P < .05$ *v* SEKI mice.

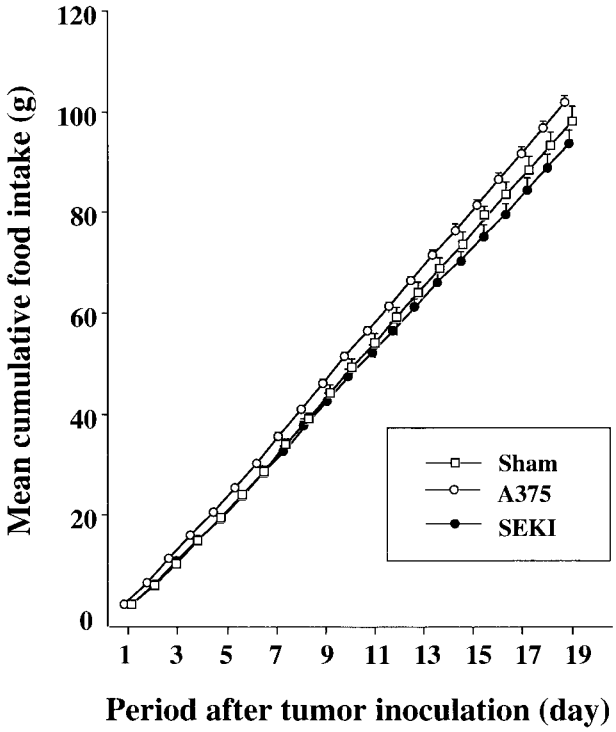

Fig 3. Mean (+SE) cumulative food intake by Sham, A375, and SEKI mice after tumor inoculation.

Plasma corticosterone and LIF concentrations are presented in Table 2. In SEKI mice, plasma LIF was already detectable on day 9 before the onset of weight reduction and increased by more than 10-fold on day 19. Plasma LIF of the A375 mice was also detectable, but the level was much lower than that of the SEKI mice. Plasma LIF was not detectable either in PF or Sham groups. Serum corticosterone concentrations were similar in all groups on days 9 and 19.

Quantitative RT-PCR for hypothalamic neuropeptide mRNA showed that NPY and CRH mRNA levels in whole hypothalamus were elevated significantly in the SEKI mice on day 19 as compared with the other 3 groups. There were no differences in MCH, orexin, and CCK mRNA levels among the 4 groups on day 19, although CCK mRNA in SEKI mice exhibited a nonsignificant trend toward reduction (Figs 4 and 5). There was no significant difference in the neuropeptide mRNA levels among groups on day 9.

In situ hybridization histochemistry revealed that NPY mRNA levels in the arcuate nucleus (ARC) were conspicuously elevated in SEKI mice (Fig 6c) as compared with Sham mice (Fig 6a), whereas the NPY mRNA signals dispersed throughout the cerebral cortex were unchanged (data not shown). Signals for CRH mRNA whose expression was restricted mainly to the paraventricular nucleus were also increased in intensity in SEKI mice, displaying densely labeled neuronal somata (Fig 6d).

DISCUSSION

The human melanoma cell line SEKI has been reported to induce severe weight loss in nude mice and the causative factor is supposed to be LIF, although other factors may also be involved.[8,9] We investigated the involvement of the central nervous system in the regulation of body weight in this cancer cachexia model by studying the gene expression of neuropeptides, known to regulate energy balance. We chose a competitive PCR technique for accurate measurement of mRNA levels.

The hypothalamic mRNA levels of neuropeptide NPY were selectively increased in the ARC of cachectic SEKI mice in comparison to A375-bearing, PF, or Sham control mice. Because food consumption of cachectic SEKI mice was similar to Sham or A375 mice, increased gene expression of NPY seems to be caused by weight reduction and not by decrease in food intake, suggesting that SEKI mice detected a state of energy deficit. A simple interpretation of the elevation of NPY mRNA is that the increase leads to enhanced synthesis of NPY to replenish the peptide stores that are depleted in association with increased NPYergic activity. If this interpretation is correct, the NPYergic system is functioning in the rodent models of cancer cachexia, because NPY acts within the brain to influence peripheral metabolic systems in a way that favors energy storage.[18] This result is surprising because previous study demonstrated that the hypothalamic concentration of NPY, release of NPY into hypothalamic interstitial fluid, and feeding response following intrahypothalamic injection of NPY were all decreased in anorectic tumor-bearing rats when compared with nontumor-bearing counterparts.[3-5] Furthermore, there was a possibility that LIF could suppress upregulation of NPY mRNA in response to weight loss, because LIF is a member of the cytokine family structurally related to ciliary neurotropic factor, which is reported to prevent the fasting-induced upregulation of NPY gene expression in the hypothalamus when administrated intracerebroventricularly.[19] Further investigation is necessary to determine whether or not the increased hypo-

Table 2. Plasma Corticosterone and LIF Concentrations

	Sham	PF	A375	SEKI
Plasma corticosterone (ng/mL)				
Day 9	132.2 ± 8.3	386.8 ± 4.0	198.9 ± 2.3	246.5 ± 6.0
Day 19	261.7 ± 73.8	257.8 ± 4.8	149.8 ± 40.9	330.5 ± 8.6
Plasma LIF (ng/mL)				
Day 9	< .008*	< .008*	.01 ± 0.007*	.81 ± .21
Day 19	< .008*	< .008*	.30 ± 0.07*	10.63 ± 1.17

*$P < .01$ *v* SEKI.

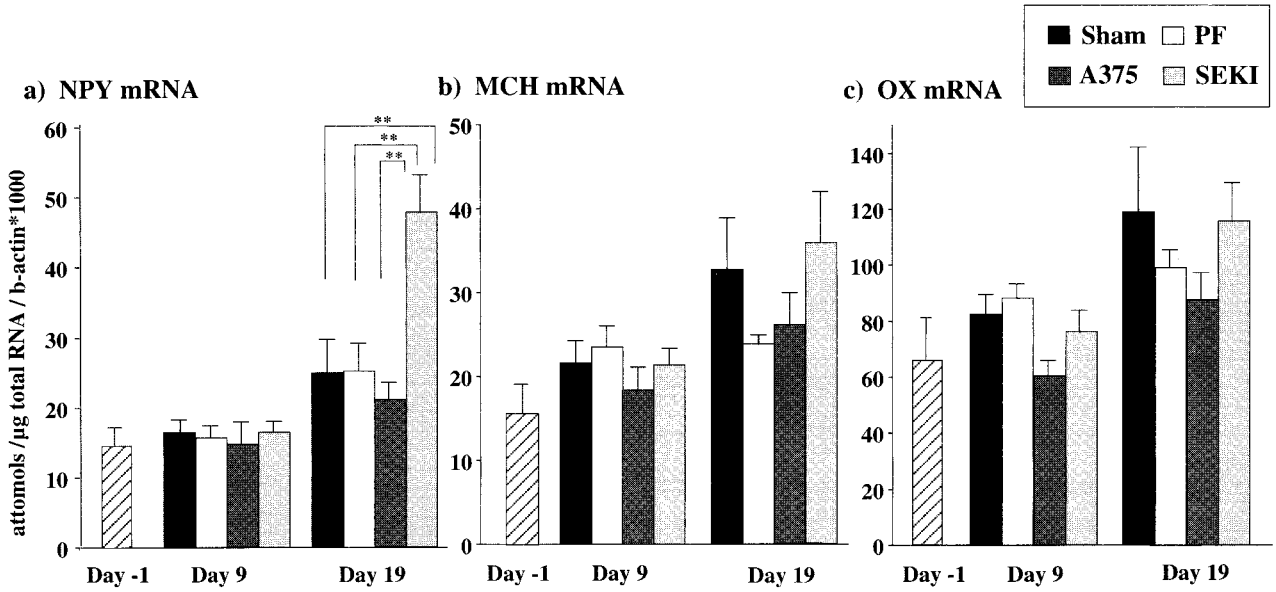

Fig 4. Mean (+SE) orexigenic neuropeptide mRNA levels measured by quantitative RT-PCR in hypothalamus taken from Sham, PF, A375, and SEKI mice. **$P < .01$ *v* SEKI mice.

thalamic NPY mRNA is truly associated with increased neuronal activity.

Although the mechanism enhancing NPY gene expression in SEKI mice is unknown, several hormones may be likely candidates for the mediators. Among these are elevated plasma glucocorticoid levels. Recently, other investigators have provided evidence that glucocorticoid receptors are localized on NPY-synthesizing neurons in the ARC,[20] and elevated levels of circulating glucocortcoids directly influence NPY gene expression.[21] Since plasma corticosterone levels of SEKI and control groups were similar in this study, however, the increase of NPY gene expression could not be accounted for by a change in the levels of this hormone. We cannot rule out the possibilities that the circulating insulin and leptin levels might have been decreased during weight reduction, and these changes might have contributed to the increase in NPY mRNA expression.[22,23]

In contrast to NPY mRNA, hypothalamic MCH and OX mRNA levels in SEKI mice were not significantly different from those in control mice. Different responses of the gene expression of orexigenic peptides in cachectic mice may reflect distinct roles of these neuropeptides in the body weight regulation. Induction of feeding by MCH or OX has been shown to be of short duration and is not as robust as NPY-induced feeding.[24,25] Because cancer cachexia involves a chronic negative energy balance, MCH and OX may not play major roles in the body weight control of cachectic mice.

Whereas NPY promotes energy storage, the effects of CRH

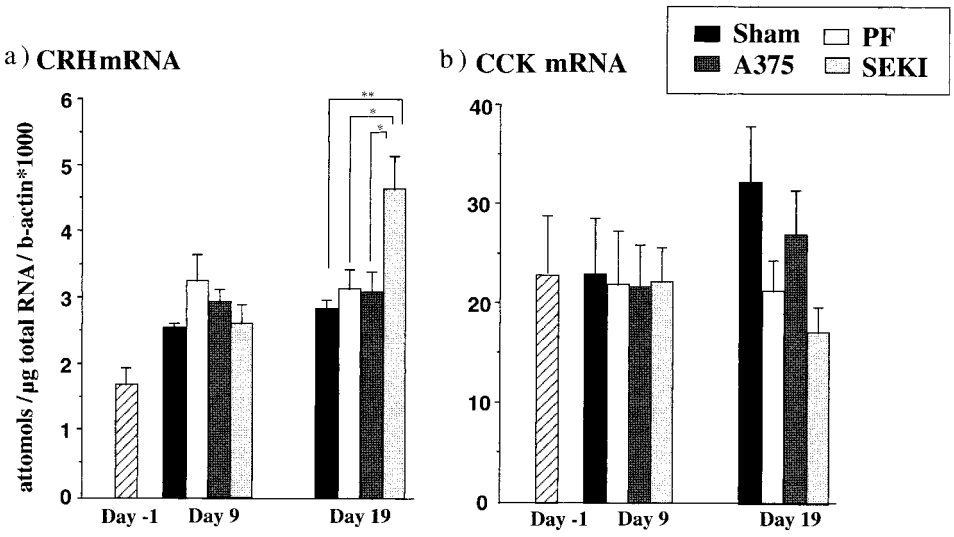

Fig 5. Mean (+SE) anorexigenic neuropeptide mRNA levels measured by quantitative RT-PCR in hypothalamus taken from Sham, PF, A375, and SEKI mice. *$P < .05$ and **$P < .01$ *v* SEKI mice.

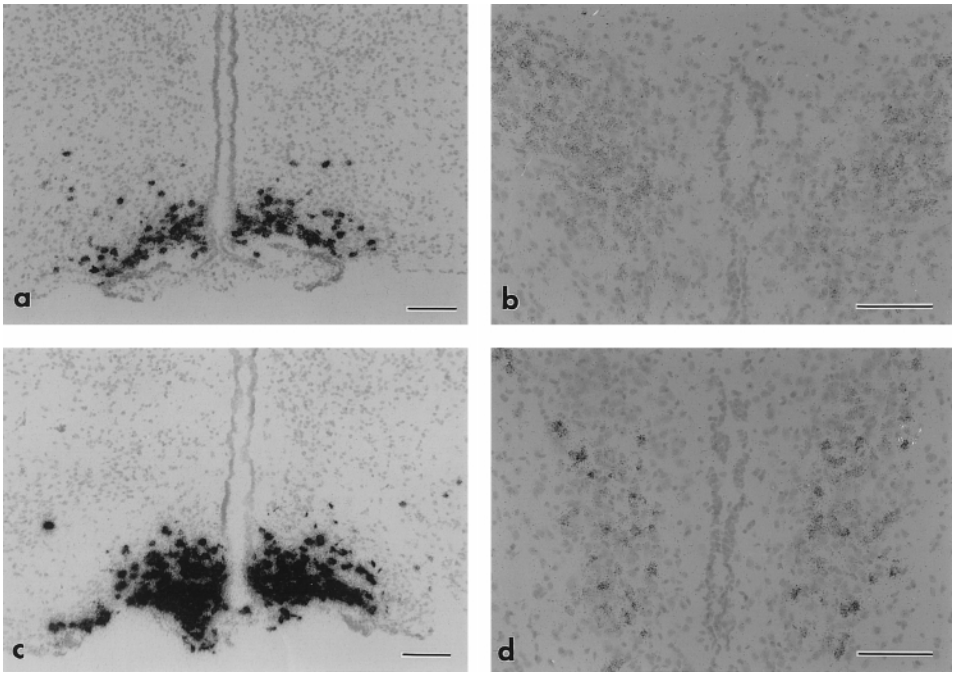

Fig 6. In situ hybridization for detection of NPY (a and c) and CRH (b and d) mRNA in sections from the hypothalamus. NPY mRNA signals in the ARC increase in intensity in SEKI mice (c) as compared with Sham mice (a). Signals for CRH mRNA in the PVN are weak and diffusely distributed in Sham mice (b), while in SEKI mice they accumulate in several neurons, possibly parvocellular in type (d). Bars represent 100 μm.

on energy balance are catabolic in nature, promoting loss of energy from the body.[26,27] Previous studies showed that continuous intracerebroventricular administration of CRH caused sustained anorexia and progressive weight loss associated with increased sympathetic activity and thereby increased thermogenesis and lipolysis.[26,27] In the present study, hypothalamic CRH mRNA levels were elevated in the tumor-bearing, cachectic mice. This response is not consistent with those observed in rats and hamsters whose hypothalamic CRH mRNA levels were decreased or unchanged after chronic food restriction.[28,29] The underlying mechanisms of an increase of hypothalamic CRH mRNA in SEKI mice remain to be elucidated. Sustained elevation of the circulating level of LIF might have stimulated the hypothalamic-pituitary-adrenal (HPA) axis and increased hypothalamic CRH mRNA in SEKI mice, because LIF has been reported to stimulate the HPA axis.[30] It is unlikely that the stress from a tumor burden is the cause of increased CRH mRNA, because plasma corticosterone levels were not significantly different among 4 groups. Moreover, the control A375-bearing mice did not show such an mRNA increase, suggesting that the tumor burden is not the major cause of increase in hypothalamic CRH mRNA in SEKI mice. However, the possibility of stress from a tumor burden is not completely excluded, because the tumor size of SEKI was larger than A375.

In summary, we demonstrated that hypothalamic NPY and CRH mRNA levels were increased in tumor-bearing SEKI mice in association with body weight reduction. These results support the notion that the NPYergic system is functioning in the rodent models of cancer cachexia, however, the role of the CRHergic system in energy homeostasis remains to be elucidated. This is the first report of the hypothalamic neuropeptide response to cachexia-inducing human cells.

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