Upregulation of Ghrelin Expression in Cachectic Nude Mice Bearing Human Melanoma Cells

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Ghrelin is a gastrointestinal peptide that stimulates food intake and growth hormone (GH) secretion. We studied the biosynthesis and secretion of ghrelin in a cancer cachexia mouse model. G361, a human melanoma cell line, was inoculated into nude mice. The body weight was reduced and the plasma concentration of interleukin-1β (IL-1β) was markedly higher in tumor-inoculated mice compared with vehicle-treated mice. Furthermore, white adipose tissue (WAT) weight, blood sugar level, and plasma concentrations of leptin and nonesterified fatty acids (NEFA) were significantly lower in tumor-inoculated mice. The plasma concentration of ghrelin increased with the progression of cachexia. The levels of both ghrelin peptide and mRNA in the stomach were also upregulated in tumor-inoculated mice. This study demonstrates that both ghrelin biosynthesis and secretion are stimulated in the long-term negative energy balance of tumor-inoculated cachectic mice. These findings suggest the involvement of ghrelin in the regulation of energy homeostasis in cancer cachexia.

CANCER CACHEXIA, a catabolic state characterized by weight loss and muscle wasting, occurs frequently in patients with end-stage neoplastic disease.[1,2] Approximately half of all cancer patients suffer from cachexia, a strong independent risk factor for mortality.[3,4] The cachexia cannot be attributed solely to appetite loss, and nutritional supplementation alone is unable to reverse the wasting process. Numerous cytokines produced by tumor cells, including leukemia-inhibitory factor (LIF), tumor necrosis factor-α, interleukin-1β (IL-1β), IL-6, and interferon γ, mediate the cachectic effect of cancer.[2,5] A variety of neuropeptides, including neuropeptide Y (NPY), melanin-concentrating hormone, orexin, melanocortin, cholecystokinin, and corticotropin-releasing hormone, regulate energy balance and metabolic signaling.[3] Alterations of these neuropeptide networks may be responsible for the development of cachectic syndrome.

Ghrelin, initially discovered from rat and human stomach as an endogenous ligand for the growth hormone (GH) secretagogue receptor,[6] is an enteric peptide that stimulates food intake[7-9] and induces adiposity.[10] Daily subcutaneous administration of ghrelin causes body weight gain and increases fat mass in mice and rats. Upon fasting, plasma ghrelin concentrations are increased in rats and humans; these levels are decreased after meals.[11-14] Ghrelin serves as an anabolic hormone produced in the stomach and may contribute to energy homeostasis in cancer cachexia. We previously demonstrated that G361, a human melanoma cell line that produces IL-1β and LIF, causes severe body weight loss in nude mice upon inoculation.[15] To investigate the involvement of ghrelin in cancer cachexia, we have studied ghrelin expression in the stomach of G361-inoculated nude mice. We also determined the gastric content of ghrelin and its plasma concentration in the early and end stages of cancer cachexia.

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MATERIALS AND METHODS

Animals

Five-week-old female BALB/c-*nu/nu* mice weighing 15 to 17 g (Charles River Japan, Atsugi, Japan) were adapted to laboratory conditions for 1 week before the start of experiments. Mice were housed individually in plastic cages containing wood chips in a temperature-controlled 23°C ± 2°C room under 12:12-hour light:dark cycles (light on at 7 AM). Animals were maintained on tap water and a breeding diet (CRF-1; Oriental Yeast, Tokyo, Japan) placed on the ground. All procedures were performed in accordance with the Japanese Physiological Society's guidelines for animal care and approved by the Miyazaki Medical College animal care committee.

Tumor Inoculation

Mice were allocated into 3 groups. Each mouse in groups 1 and 2 was inoculated subcutaneously in both flanks with either 1×10^7 G361 human melanoma cells (hereafter referred to as "G361-1 and G361-2 mice") or vehicle alone (hereafter referred to as "vehicle mice"). Their body weights were measured every day at 9 AM. G361-1 mice (n = 10) were euthanized with sodium pentobarbital anesthesia 7 days after inoculation, and G361-2 (n = 7) and vehicle mice (n = 8) were killed 13 days after inoculation. Blood was collected from the heart into polypropylene tubes containing EDTA·2Na. The blood sugar level was measured using ANTSENSE II (DAIKIN, Tokyo, Japan). The stomach was removed and divided into 2 portions; one half was utilized for peptide quantification and the other was immediately homogenized with TRIzol (Life Technologies, Frederick, MD) for RNA isolation. The tumor and white adipose tissue (WAT) surrounding the kidney and uterus were removed and weighed.

Plasma Analyses

Plasma concentrations of IL-1β, leptin, and nonesterified fatty acid (NEFA) were measured with a human IL-1β immunoassay kit (R&D Systems, Minneapolis, MN), a Leptin/Mice enzyme-linked immunosorbent assay (ELISA) kit (SEIKAGAKU, Tokyo, Japan), and a

Determiner NEFA assay kit (Kyowa Medex, Tokyo, Japan), respectively.

Ghrelin Quantification in Mice

Stomach. Ghrelin content in the stomach was measured by radioimmunoassay (RIA) specific for ghrelin as described.[16] In brief, approximately 50 mg of mouse stomach was boiled for 10 minutes in a 100-fold volume of water to inactive intrinsic proteases. After cooling to 4°C, CH_3COOH and hydrochloric acid (HCl) were added to final concentrations of 1 mol/L and 20 mmol/L, respectively. The stomach was then homogenized in a Polytron for 1 minute. After centrifugation, the supernatant, equivalent to 3 mg wet weight, was lyophilized and subjected to RIA. Both a standard peptide solution and the diluted sample (100 μL) were incubated for 24 hours with 100 μL antighrelin (13–28) antiserum (final dilution 1/20,000). Following addition of a tracer solution ([^{125}I-Tyr^0]ghrelin (13–28), 17,000 cpm/100 μL), the mixture was again incubated for 24 hours. Bound and free ligand were separated using a secondary antibody (200 μL). Samples were assayed in duplicate; all procedures were performed at 4°C. The antiserum recognized acylated and nonacylated ghrelin on an equimolar basis. The RIA system specifically detected both ghrelin molecules, a finding that was confirmed by high-performance liquid chromatography coupled with the RIA.[16] The limit of detection of the assay was 12 fmol/tube for mouse ghrelin. The respective intra- and interassay coefficients of variation were 3.7% and 3.3% at 50% binding. More than 95% of ghrelin was recovered from the stomach extract.

Plasma. Plasma from each of the 3 mouse groups was pooled group wise. The pooled plasma samples (0.4 mL) were diluted with equal volumes of 0.9% saline and then applied to a Sep-Pak C_{18} cartridge (Waters, Milford, MA) equilibrated with 0.9% saline. The cartridge was washed with 0.9% saline and 10% acetonitrile (CH_3CN) solution containing 0.1% trifluoroacetic acid (TFA). Absorbed peptides were eluted with 60% CH_3CN solution containing 0.1% TFA, lyophilized, and then subjected to RIA. The recovery of ghrelin added to the mouse plasma sample in the extraction done with a Sep-Pak C_{18} cartridge was more than 92%.

Northern Blot Analysis

Total RNA was extracted from mouse stomach using TRIzol. Ghrelin mRNA was quantified by Northern blot analysis. Ten micrograms of total RNA were denatured using 2 mol/L glyoxal and 50% dimethyl sulfoxide. Following 1.2% agarose gel electrophoresis, the sample was transferred to a Zeta Probe membrane (Bio-Rad Laboratories, Richmond, CA). The probes used for hybridization recognize full-length ghrelin cDNA and a 0.2-kb cDNA fragment of glycerol-3-phosphate dehydrogenase (G3PDH).[12] Membranes were first hybridized for 1.5 hours at 42°C in 6 × SSPE (900 mmol/L NaCl, 60 mmol/L NaH_2PO_4, 7 mmol/L EDTA, pH 7.4) containing 40% formamide, 5 × Denhardt's solution, 0.5% sodium dodecyl sulfate (SDS), and 0.1 mg/mL denatured salmon sperm DNA. Membranes were then hybridized for 24 hours at 42°C in a solution containing the two ^{32}P-labeled cDNA probes. The RNA blot was immersed in 2 × SSC (300 mmol/L NaCl, 30 mmol/L sodium citrate, pH 7.0)/0.1% SDS for 20 minutes at 58°C, followed by an incubation in 1 × SSC/0.1% SDS for 40 minutes. Hybridized signals were quantified using a Fuji Bio-image analyzer (BAS 2000, Fuji Film, Tokyo, Japan).

Statistical Analysis

All data are presented as means ± SEM. Comparisons between groups were performed using the unpaired *t* test. *P* values < .05 were considered significant.

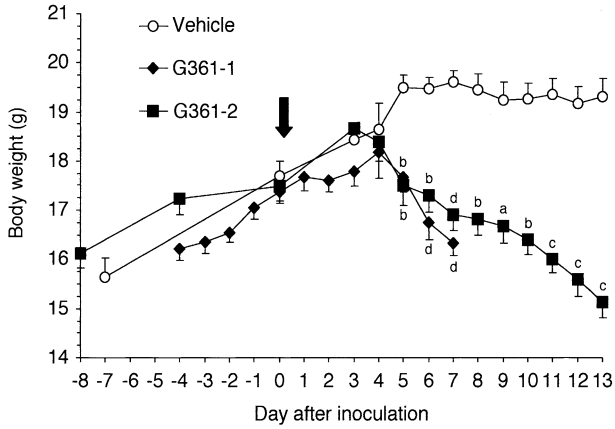

Fig 1. Body weight of G361- (G361-1 and G361-2) or vehicle-inoculated nude mice. G361-1 mice were euthanized 7 days after inoculation, and G361-2 and vehicle mice were euthanized 13 days after inoculation. An arrow indicates the day when either tumor cells or vehicle was inoculated. Data represents means ± SEM. $^aP < .01$, $^bP < .001$, $^cP < .0001$, $^dP < .00001$ *v* vehicle mice.

RESULTS

Body Weight of Tumor-Inoculated Mice

The mean body weights of G361-1 and G361-2 mice began to decrease 5 and 4 days, respectively, after tumor inoculation (Fig 1). Five days after inoculation, the body weights of both G361-1 and G361-2 mice were significantly lower than that of vehicle-treated control mice. There was no significant difference in body weight between G361-1 and G361-2 mice. The ratios of the body weight at sacrifice to the peak body weight were 88% for G361-1 mice and 80% for G361-2 mice, respectively. The individual tumor weights in all mice were less than 0.2 g each, and there was no significant difference in the tumor weight between G361-1 and G361-2 mice. The plasma concentrations of IL-1β in both G361-1 and G361-2 mice were markedly higher than that of vehicle mice (Fig 2).

WAT Weight, Blood Sugar, and Plasma Concentrations of Leptin and NEFA

WAT weight, blood sugar level, and plasma concentrations of leptin and NEFA are shown in Table 1. In both G361-1 and G361-2 mice, the values of all measurements were significantly lower than those in vehicle mice. In G361-2 mice, the mean values of all measurements were lower than those in G361-1 mice; in particular, the blood sugar level was significantly lower.

Ghrelin Peptide and mRNA Levels

Plasma from control vehicle, G361-1, and G361-2 mice was pooled groupwise before assays were performed because the plasma volume from 1 mouse was too small to be subjected to ghrelin RIA. The plasma concentrations of ghrelin in G361-2 and G361-1 mice were higher than that of vehicle mice (Fig 3). The plasma concentration of ghrelin in G361-2 was 2.4-fold higher than that of G361-1 mice. Similarly, the ghrelin content in the stomachs of G361-2 mice was significantly higher than

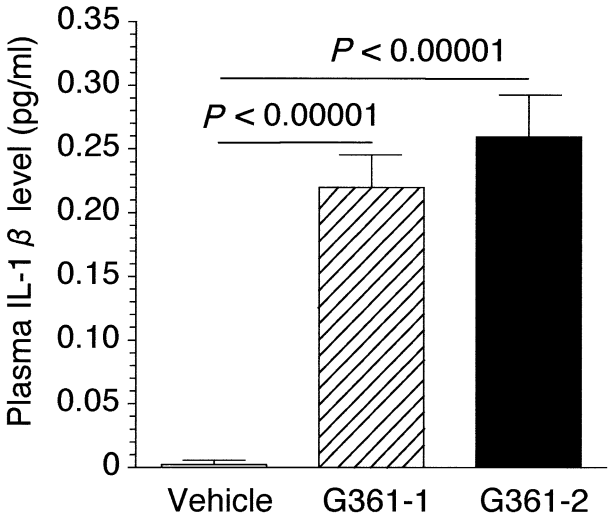

Fig 2. Plasma concentrations of IL-1β.

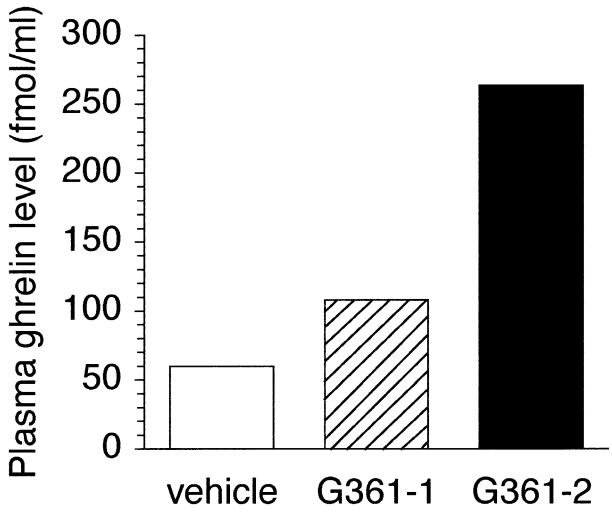

Fig 3. Plasma concentrations of ghrelin.

those of vehicle and G361-1 mice (Fig 4A), and the gastric ghrelin content of G361-1 mice tended to be higher than that of vehicle mice ($P = .08$). Ghrelin mRNA levels of both G361-1 and G361-2 mice were significantly higher than that of vehicle mice, and the mRNA level of G361-2 mice was significantly higher than that of G361-1 mice (Fig 4B).

DISCUSSION

Ghrelin is the first neuroenteric peptide shown to act as a starvation-signaling molecule in the periphery, stimulating feeding after peripheral administration.[6-14] Ghrelin secretion is upregulated under conditions of negative energy balance and downregulated in the setting of positive energy balance. Plasma ghrelin concentration is increased in cancer anorexia model rats bearing adenocarcinoma cell,[17] human patients with anorexia nervosa,[14,18-20] and cachectic patients with cardiac heart failure.[21] However, little is known regarding alterations of ghrelin biosynthesis and secretion in a state of long-term negative energy balance, such as a cachexia-anorexia syndrome. In this study, a G361-inoculated cachexia mouse model was used to investigate the alteration of ghrelin expression and secretion with the progress of cachexia during its early (7 days after inoculation) and late (13 days) stages.

G361 cells induce severe cachexia by producing several cytokines, including IL-1β and LIF.[15] In the G361-inoculated mice, plasma concentration of IL-1β was elevated compared with that of the vehicle-treated group. The increased level of IL-1β might be one of the causes of cachexia, for IL-1β is known to induce severe weight loss.[22,23] As cachexia worsened, the symptoms of negative energy balance, such as fat loss and hypoglycemia, developed. Plasma leptin concentration correlates with the amount of adipose tissue.[24] The decrease in plasma leptin concentration in tumor-inoculated mice might be due to a reduction in the amount of adipose tissue, which produces leptin. Plasma NEFA concentration usually increases in the initial phase of anorexia because of lipolytic compensation for energy deficiency,[25] but in this study, it was decreased in both early- (G361-1) and late-stage (G361-2) G361-inoculated cachectic mice. The weight of adipose tissue was extremely low in the 2 groups of G361-inoculated mice. Lower plasma NEFA concentration in these mice may be due to decreased adipose tissue mass.

We established 2 ghrelin-specific RIAs; one recognizes the octanoyl-modified portion and another the C-terminal portion of ghrelin.[26] The antibody raised against ghrelin [13-28] equally measures desoctanoylated and octanoylated ghrelin, and it does not distinguish how much of each molecular form is present in the samples. We measured total immunoreactivity of ghrelin molecules by using the ghrelin RIA recognizing its C-terminal portion. Plasma ghrelin concentration, gastric ghrelin content, and ghrelin mRNA level all increased in both early- and late-stages G361-inoculated cachectic mice. These increments may result from stimulation of both biosynthesis and secretion of ghrelin in a long-term negative energy balance state, such as cachexia. In addition, ghrelin levels in the stomach and plasma were higher in late-stage G361-inoculated mice than in early-stage animals, suggesting that ghrelin biosynthesis and secretion became elevated as cachectic symptoms worsened. Increased ghrelin levels in cachectic mice might reflect a physiologic adaptation to negative energy balance. Body weight in cachectic mice decreased, despite elevated ghrelin levels in the stomach and plasma. This may be due to the

Table 1. White Adipose Tissue Weight, Blood Sugar, and Plasma Concentrations of Leptin and Nonesterified Fatty Acid in Mice

	WAT (mg)	BS (mg/dL)	Leptin (pg/mL)	NEFA (μEq/L)
Vehicle (n = 8)	121 ± 32	201 ± 9	1,593 ± 264	427 ± 42
G361-1 (n = 10)	16 ± 2*	163 ± 9*	618 ± 168*	226 ± 30†
G361-2 (n = 7)	7 ± 5*	89 ± 8‡§	361 ± 54†	133 ± 39†

NOTE. Data represent the mean ± SEM.

*$P < .01$, †$P < .001$, ‡$P < .000001$ *v* vehicle mice and §$P < .0001$ *v* G361-1 mice.

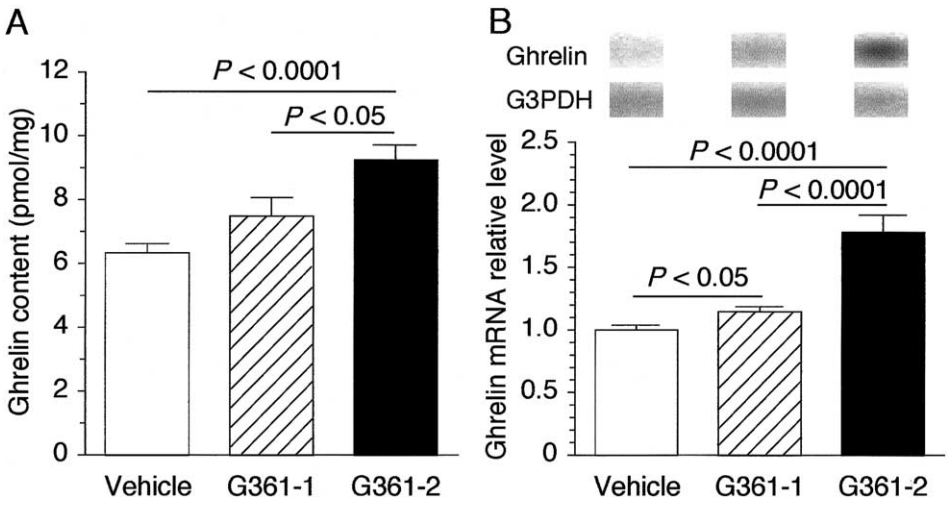

Fig 4. (A) Ghrelin content in the stomach. (B) Upper: representative Northern blot analysis patterns of ghrelin mRNA in the stomach. Lower: ghrelin mRNA levels relative to G3PDH levels.

effects of cachexia-inducing factors produced by G361 melanoma cells that outweigh the anabolic effect of ghrelin.

The stomach is a major source of circulating ghrelin in humans and rats.[6,16,18] Ghrelin-producing endocrine cells, which are most abundant in the oxyntic mucosa of both species, account for about 20% of the oxyntic gland endocrine cell population.[16,27] Ghrelin expression and secretion are affected by energy imbalance.[10-14,17-20] Taken together, there may be a system in ghrelin-producing cells in the stomach that responds to alterations of energy homeostasis. The molecular signals that regulate ghrelin biosynthesis and secretion need to be elucidated.

In summary, the present study demonstrated that ghrelin biosynthesis and secretion are upregulated with the progression of cachexia in tumor-bearing mice. Considering ghrelin's effect on positive energy balance, elevated ghrelin may represent a compensatory mechanism under catabolic-anabolic imbalance in cancer cachexia.

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