New cancer cachexia rat model generated by implantation of a peritoneal dissemination-derived human stomach cancer cell line

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Am J Physiol Endocrinol Metab 306: E373–E387, 2014. First published December 17, 2013; doi:10.1152/ajpendo.00116.2013.—Cancer cachexia (CC), a syndrome characterized by anorexia and body weight loss due to low fat-free mass levels, including reduced musculature, markedly worsens patient quality of life. Although stomach cancer patients have the highest incidence of cachexia, few experimental models for the study of stomach CC have been established. Herein, we developed stomach CC animal models using nude rats subcutaneously implanted with two novel cell lines, i.e., MKN45c185, established from the human stomach cancer cell line MKN-45, and 85As2, derived from peritoneal dissemination of orthotopically implanted MKN45c185 cells in mice. Both CC models showed marked weight loss, anorexia, reduced musculature and muscle strength, increased inflammatory markers, and low plasma albumin levels; however, CC developed earlier and was more severe in rats implanted with 85As2 than in those implanted with MKN45c185. Moreover, human leukemia inhibitory factor (LIF), a known cachectic factor, and hypothalamic orexigenic peptide mRNA levels increased in the models, whereas hypothalamic anorexigenic peptide mRNA levels decreased. Surgical removal of the tumor not only abolished cachexia symptoms but also reduced plasma LIF levels to below detectable limits. Importantly, oral administration of rikkunshito, a traditional Japanese medicine, substantially ameliorated CC-related anorexia and body composition changes. In summary, our novel peritoneal dissemination-derived 85As2 rat model developed severe cachexia, possibly caused by LIF from cancer cells, that was ameliorated by rikkunshito. This model should provide a useful tool for further study into the mechanisms and treatment of stomach CC.

Cancer cachexia; leukemia inhibitory factor; rikkunshito; stomach cancer model; anorexia

Cancer cachexia, a multifactorial syndrome characterized by anorexia and the loss of body weight, adipose tissue, and skeletal muscle, is observed in 80% of advanced cancer patients and accounts for at least 20% of cancer-related deaths (20, 35, 42). This syndrome causes not only poor quality of life (QOL) but also poor responses to chemotherapy, highlighting the need for improved cancer cachexia treatments. Weight loss, the most prominent clinical feature of cachexia, is observed in 30–80% of cancer patients, depending on tumor type. For example, weight loss occurs at a very high frequency (83%) in stomach and pancreatic cancer patients but is less prominent in patients with breast cancer, acute nonlymphocytic leukemia, and sarcomas (35). Although cachexia strongly impacts the success of therapeutic treatments, the mechanisms underlying this syndrome are not fully understood. Stomach cancer patients in particular have the highest incidence of cachexia; however, few experimental models for the study of stomach cancer cachexia have been established (4, 14, 66).

A useful cachexia model must meet three of the following five diagnostic criteria in addition to weight loss: anorexia, decreased muscle strength, fatigue, low fat-free mass (FFM) index, and abnormal biochemistry (anemia, increased inflammatory markers, and low serum albumin) (14). Moreover, Argilés et al. (1) reported that two specific indicators, anorexia and metabolic disturbances, should be identified before arriving at a diagnosis of cachexia-associated weight loss. Although body weight maintenance is the most important end point of any cachexia treatment, body composition and QOL should also be monitored (1). Accordingly, anorexia and body composition are very important in both the diagnosis and treatment of cachexia and should be present in any experimental model designed to study this syndrome.

To address the need for an experimental stomach cancer cachexia animal model, we previously screened 15 human stomach cancer cell lines for their ability to induce weight loss in mice after subcutaneous implantation (63). Among the cell lines that were screened, only the MKN-45 cell line induced body weight loss, with an incidence of 40% in tumor-bearing mice (63). On the basis of these findings, we established two novel cell lines from MKN-45 cells: MKN45c185 and 85As2 (63). Implantation of MKN45c185 cells induces body weight loss in mice with 100% efficiency. The 85As2 cell line, derived from peritoneal metastasis of orthotopically implanted MKN45c185 cells, has a strong capacity to induce peritoneal dissemination and body weight loss (100% efficiency) in mice. Anorexia is a key factor in both the diagnosis and treatment of cachexia. Appetite facilitation reinforces physical strength and improves QOL. Thus, anorexia is very important for the evaluation of cachexia. In our previous mouse model, evaluation of cachexia based on weight loss was possible, whereas anorexia could not be used to assess cachexia because of instability in the reduction of food consumption (63). There-

Address for reprint requests and other correspondence: K. Terawaki, Div. of Cancer Pathophysiology, National Cancer Center Research Institute, 5-1-1 Tsukiji, Chuo-ku, Tokyo 104-0045, Japan (e-mail: kiterawak@ncc.go.jp).
fore, our mouse cachexia model was not suitable to evaluate drug efficacy or mechanisms of cachexia-associated anorexia. To address this problem, we aimed to establish two novel stomach cancer cachexia models by implanting MKN45cl85 and 85As2 cell lines into nude rats. We determined the usefulness of these cancer cachexia models in evaluating anorexia, body composition changes (including low FFM), and weight loss. Moreover, body composition changes, including low FFM, are useful in elucidating the mechanisms of anorexia associated with stomach cancer cachexia. To investigate the underlying mechanisms of cachexia in these models, plasma levels of cytokines known to be involved in cancer cachexia development, such as interleukin (IL)-1, IL-6, tumor necrosis factor (TNF)α, and leukemia inhibitory factor (LIF) (13, 19, 40, 58), were also evaluated. Because the hypothalamus is a key regulator of energy homeostasis and a major site for the integration of metabolic signals in the central nervous system, the expression of hypothalamic feeding-regulating peptides was determined. In addition, the expression of the muscle-specific E3 ubiquitin ligases atrogin-1/MAFbx and muscle RING finger 1 (MuRF-1), which are important mediators of skeletal muscle loss, was also evaluated (6, 25).

To assess the efficacy of our novel stomach cancer cachexia rat models in evaluating treatment outcomes, we examined the impact of rikkunshito therapy on cachexia-associated symptoms in these models. Rikkunshito has been approved by the Ministry of Health, Labor, and Welfare of Japan and is widely prescribed as a remedy for various gastrointestinal syndromes, such as anorexia, dyspepsia, and gastritis (29, 44). Rikkunshito was found to improve anorexia symptoms in a double-blind study of patients with functional dyspepsia (29). Additionally, increasing evidence from experimental animal models has shown that rikkunshito ameliorates several types of anorexia (23, 24, 57). It has been reported that with rikkushito there is an increase in the secretion of the orexigenic hormone ghrelin by the inhibition of 5-HT2B/2C receptors, an enhancement of ghrelin receptor (GHSR) signaling efficiency, and a facilitation of gastric emptying and gastric adaptive relaxation, all of which may contribute to ameliorate anorexia (24, 32, 57, 60, 61). Herein, we examined specifically the efficacy of rikkunshito in ameliorating anorexia symptoms in our novel cancer cachexia rat models.

**MATERIALS AND METHODS**

**Animal experiment.** Six-week-old male F344/Ncl-rlnu/rnu rats (Clea-Japan, Tokyo, Japan) were housed individually under a 12:12-h light-dark cycle (lights on at 0800) at a constant temperature and humidity, with ad libitum access to food and water. Rats were allowed to acclimate to laboratory conditions for 2 wk prior to experimentation. All studies were performed according to the Guidelines for Animal Experiments drafted for the Committee for Ethics in Animal Experimentation of the National Cancer Center and approved by the committee (approval nos. T09-050-M02 and T09-050-C04), and they met the ethical standards required by the law and the guidelines concerning experimental animals in Japan.

**Cell lines and culture conditions.** MKN45cl85 and 85As2 cell lines were established from the human stomach MKN-45 cancer cell line, as described previously (63). Cells were maintained in RPMI 1640 medium (Nacalai Tesque, Kyoto, Japan) supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA), 100 IU/ml penicillin G sodium, and 100 µg/ml streptomycin sulfate (Nacalai Tesque) under a 5% CO2 and 95% air atmosphere at 37°C.

**Tumor cell implantation.** MKN45cl85 and 85As2 cells were harvested from subconfluent cultures after brief exposure to 0.25% trypsin and 0.2% ethylenediaminetetraacetic acid. Cells were washed once in serum-free medium and resuspended in phosphate-buffered saline. Anesthetized rats were subcutaneously inoculated with either 1 × 10^7 cells/site (tumor-bearing rats) or saline alone (non-tumor-bearing control rats) in the left and right flanks. The major and minor tumor axes were measured, and the tumor volume was estimated using the following equation: tumor volume (cm³) = major axis (cm) × minor axis (cm) × minor axis (cm) × 1/2, and tumor volume was converted to tumor weight (mg/mm³) (14, 17, 66).

**Rat model characterization.** Body weight and composition, organ tissue weight, and food and water consumption, and grip strength were measured weekly. Body composition was determined using the ImpediVET Bioimpedance Spectroscopy device (ImpediMed, Brisbane, Australia) to accurately measure FFM, fat mass (FM), and total body water (TBW) (52). Blood collected from the abdominal aorta was centrifuged (3,000 rpm, 10 min), and the plasma was stored at −80°C until analysis. Organ tissues were immediately dissected and weighed. Gastrocnemius muscle tissue was fixed with 10% formalin and embedded in paraffin for histological analysis. Cross-sectional area, perimeter, Feret diameter, and minimum Feret diameter were calculated using Image J software (15). Grip strength was measured using a grip strength meter for rats and mice (MK-380CM/F; Muromachi Kidai, Tokyo, Japan).

**Tumor removal experiment.** Anesthetized rats were subcutaneously inoculated with either 85As2 1 × 10^7 cells/site (tumor-bearing rats) or saline alone (non-tumor-bearing control rats) in the left and right flanks. Tumor growth, body weight, and food and water intake were measured weekly. Cancer cachexia clearly developed in tumor-bearing rats 2 wk after implantation. After that, tumor-bearing cancer cachectic rats were divided into two groups; one group was not treated and the other group had their tumors removed. Tumors were removed with surgical scissors, and the incision was closed using a surgical needle and sutures under anesthesia. The body composition was measured at weeks 0 (before tumor implantation), 2 (before tumor removal), and 4 (after tumor removal). Grip strength was measured, and the plasma and gastrocnemius muscle were collected 5 wk after implantation.

**Real-time polymerase chain reaction.** Real-time polymerase chain reaction (PCR) was performed as described previously (56). Briefly, the hypothalamic area was dissected on an ice-cold metal plate, and total RNA was isolated using an Isogen kit (Nippon Gene, Tokyo, Japan) according to the manufacturer’s instructions. First-strand cDNA was reverse transcribed from 5 µg of total RNA using the SuperScript First-Strand Synthesis System (Invitrogen, Carlsbad, CA) in a final volume of 100 µl. Diluted cDNA (2 µl) was amplified in a rapid thermal cycler (LightCycler; Roche Diagnostics, Barcelona, Spain) using LightCycler 480 SYBR Green I Mastermix (Roche, Barcelona, Spain) and the following primers: atrogin-1/MAFbx 5'-GAA GAC CGG CTA C TG G AA-3' (forward) and 5'-ATC AAT GGC TAG AGT ACC A-3' (reverse), forkhead box O1 (FoxO-1) 5'-GCA TCC TT G-3' (forward) and 5'-ATG GGA TGC TAC A-3' (reverse), Foxo-4 5'-GCA TCC TT G-3' (forward) and 5'-ATG GGA TGC TAC A-3' (reverse), MuRF-1 5'-GCT CTG GAG TAG GGA TGC-3' (forward) and 5'-AGG ACT CCT GCC GCA ACC C-3' (reverse), and 5'-AGG ACT CCT GCC GCA ACC C-3' (forward) and 5'-AGG ACT CCT GCC GCA ACC C-3' (reverse).

**Grip strength.** Grip strength was measured using a grip strength meter for rats and mice (MK-380CM/F; Muromachi Kidai, Tokyo, Japan).

**Histology.** Tumor-bearing rats were sacrificed 2 wk after implantation. Tumor-bearing rats were divided into two groups; one group was not treated and the other group had their tumors removed. Tumors were removed with surgical scissors, and the incision was closed using a surgical needle and sutures under anesthesia. The body composition was measured at weeks 0 (before tumor implantation), 2 (before tumor removal), and 4 (after tumor removal). Grip strength was measured, and the plasma and gastrocnemius muscle were collected 5 wk after implantation. Grip strength was measured using a grip strength meter for rats and mice (MK-380CM/F; Muromachi Kidai, Tokyo, Japan).
Cycler 480 software to analyze the exponential phase of amplification and the melting curve as recommended by the manufacturer. The amount of target mRNA in the experimental group relative to that in the control group was determined from the resulting fluorescence and threshold values (CT) using the $2^{-\Delta\Delta CT}$ method (37).

**Cytokine measurements.** Plasma levels of human IL-1β, IL-6, IL-8, TNFα, and LIF were measured using the Luminex Multiplex Assay (Affymetrix, Billerica, MA) (18). Rat IL-1β, IL-6, TNFα, keratinocyte-derived chemokine (KC), and interferon (IFN)γ plasma levels were measured using the Procarta Cytokine Assay Kit (Affymetrix). Plasma α1-acid glycoprotein and albumin levels were measured using a rat α1-acid glycoprotein enzyme-linked immunosorbent assay (ELISA) kit (Immunology Consultants Laboratory, Newberg, OR) and a rat albumin ELISA kit (Shibayagi, Gunma, Japan), respectively. Human cytokine levels were also measured in MKN45c85 and 85As2 cell culture supernatants ($5 \times 10^5$ cells/well) at 24 and 48 h.

**In situ hybridization.** In situ hybridization was performed as described previously (55). Briefly, frozen 12-μm-thick coronal brain sections were prepared in a cryostat at −20°C, thawed, and mounted onto gelatin/chrome alum-coated slides. The paraventricular nucleus...
Table 1. Body, tumor, muscle, fat, and organ weights in nude rats 4 wk after implantation with different 85As2 cell concentrations

<table>
<thead>
<tr>
<th>Control</th>
<th>1 × 10⁶ 85As2 Cells</th>
<th>1 × 10⁷ 85As2 Cells</th>
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<tbody>
<tr>
<td>TW</td>
<td>0.00 ± 0.00</td>
<td>5.19 ± 1.54*</td>
</tr>
<tr>
<td>TV</td>
<td>0.00 ± 0.00</td>
<td>3.77 ± 1.27**</td>
</tr>
<tr>
<td>BW</td>
<td>259.62 ± 5.84</td>
<td>219.78 ± 8.98***</td>
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<tr>
<td>%control</td>
<td>100.0 ± 2.3</td>
<td>84.7 ± 3.5***</td>
</tr>
<tr>
<td>BW − CTW</td>
<td>259.62 ± 5.84</td>
<td>216.01 ± 10.18***</td>
</tr>
<tr>
<td>%control</td>
<td>100.0 ± 2.3</td>
<td>83.2 ± 3.9***</td>
</tr>
<tr>
<td>BW − TW</td>
<td>259.62 ± 5.84</td>
<td>214.59 ± 10.39***</td>
</tr>
<tr>
<td>%control</td>
<td>100.0 ± 2.3</td>
<td>82.7 ± 4.0***</td>
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<tr>
<td>Peak BW</td>
<td>226.89 ± 6.06</td>
<td>206.63 ± 7.15</td>
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<tr>
<td>%peak BW</td>
<td>94.4 ± 2.7</td>
<td>88.0 ± 3.5†</td>
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### Muscle weights

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<tbody>
<tr>
<td>Greater pectoral</td>
<td>2.67 ± 0.11</td>
<td>1.88 ± 0.10***</td>
</tr>
<tr>
<td>Gastrocnemius</td>
<td>1.37 ± 0.04</td>
<td>1.17 ± 0.04***</td>
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<tr>
<td>Tibialis</td>
<td>0.54 ± 0.02</td>
<td>0.42 ± 0.03***</td>
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<tr>
<td>Soleus</td>
<td>0.08 ± 0.01</td>
<td>0.07 ± 0.00*</td>
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### Fat weights

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<tbody>
<tr>
<td>Epididymis</td>
<td>3.78 ± 0.19</td>
<td>2.73 ± 0.36*</td>
</tr>
<tr>
<td>Perirenal</td>
<td>2.58 ± 0.28</td>
<td>1.53 ± 0.45</td>
</tr>
<tr>
<td>Mesentery</td>
<td>1.41 ± 0.13</td>
<td>0.95 ± 0.29</td>
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### Organ weights

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<tbody>
<tr>
<td>Liver</td>
<td>10.36 ± 0.33</td>
<td>7.69 ± 0.56**</td>
</tr>
<tr>
<td>Spleen</td>
<td>0.63 ± 0.03</td>
<td>0.48 ± 0.02**</td>
</tr>
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</table>

Data are expressed as the mean ± SE of 5 rats; all weight data are expressed in g. TW, tumor weight; TV, tumor volume; BW, body weight; CTW, converted tumor weight. Rats were implanted subcutaneously with either 85As2 cells (1 × 10⁶ or 1 × 10⁷ cells/site) or saline alone in both flanks. TW was estimated using the following equation: TV (cm³) = 0.5 × TW (mg) / CTW (g). BW comparisons between control group and 85As2 groups at 4 wk after implantation showed the following relationship: %control (%) = BW (control) / BW (at 4 wk after implantation) × 100. Differences between groups were evaluated using Student’s t-test or Welch’s t-test. *P < 0.05 vs. the control group; **P < 0.01 vs. the control group; ***P < 0.001 vs. the control group.

Respiratory metabolism. Oxygen consumption was measured with an O₂/CO₂ metabolism-measuring system (MK-5000RQ; Muramachi Kikai, Tokyo, Japan) (33, 45). Each rat was kept unrestrained in a sealed chamber with an airflow of 0.5 l/min at 25°C for 20 h without food. Air was sampled every 3 min, and oxygen consumption (V˙O₂) was measured with an infra-red gas analyser. Respiratory quotient (RQ) was calculated as dividing VCO₂ by V˙O₂.

Palliative therapeutic studies using rikkunshito. Rikkunshito was manufactured by Tsumura (Tokyo, Japan) by spray-drying a hot water solution of powdered plant extract with xanthan gum and was dissolved in 3.5% glucose as a 5% solution for oral administration. The daily dose of rikkunshito was 1.5 ml/kg once daily for 14 days after weekly tumor cell inoculation. The number of dead rats was recorded daily, and the survival time was expressed as the number of days with no weight loss. The mean survival time, maximum survival time, and 50% survival time were determined graphically. Differences between groups were evaluated using a Mann-Whitney U test.

Fig. 2. Effects of 85As2 cell implantation at different concentrations on body weight (A), tumor volume (B), food intake (C), water intake (D), FFM (E), FM (F), and TBW (G) in nude rats. Rats were inoculated sc with 85As2 cells (1 × 10⁶ or 1 × 10⁷ cells/site) or saline (control) in both flanks at week 0. Each data point represents the mean ± SE of 5–10 rats (2–4 wk: 5 rats; 4–4 wk: 5 rats). A: body weight; B: tumor volume; C: food intake; D: FFM; E: FM; F: TBW; G: TBW; H: food intake; I: FFM; J: FM; K: TBW; L: food intake. *P < 0.05 vs. the control group. Differences between groups were evaluated using a Student’s t-test or Welch’s t-test. *P < 0.05 vs. the control group.
extract from the following eight crude drugs to form a powdered extract: Atractylodis lanceae rhizoma (4.0 g), Ginseng radix (4.0 g), Pinelliae tuber (4.0 g), Hoelen (4.0 g), Zizyphi fructus (2.0 g), Aurantii nobilis pericarpium (2.0 g), Glycyrrhiza radix (1.0 g), and Zingiberis rhizoma (0.5 g). The powdered rikkunshito extract was obtained from Tsumura. For oral administration into the stomach using a disposable sonde, rikkunshito was dissolved in distilled water (0.5 g). The powdered rikkunshito extract: Atractylodis lanceae rhizoma (4.0 g), Pinelliae tuber (4.0 g), Zizyphi fructus (2.0 g), Hoelen (4.0 g), Zingiberis rhizoma (0.5 g) was administered orally twice daily at 1,000 mg·kg⁻¹·day⁻¹ for 7 days (from days 0 to 6). The tumor-bearing control group was administered DW (10 ml/kg) over the same period. Non-tumor-bearing rats (control + DW group) were also administered DW over the same period. Tumor growth was measured weekly. Body weight and food and water intake were measured weekly until day 0 and were measured daily thereafter. Food and water intake data after rikkunshito or DW administration are expressed as the daily, cumulative value from days 0 to 7 or average value from days 2 to 7, and body weight data are expressed as body weight minus converted tumor weight. Body composition was measured on days −14 (before tumor implantation), 0 (before administration), and 6 (after administration). Rats were anesthetized with isoflurane on day 7, and muscle and adipose tissues were immediately dissected and weighed.

Statistical analyses. All data are expressed as means ± SE. Differences between groups were evaluated using the Student’s t-test, paired t-test, Welch’s t-test, Mann-Whitney U-test, one-way analysis of variance followed by a post hoc Dunn’s multiple comparison test, or Kruskal-Wallis test followed by a post hoc Dunn’s multiple comparison test. A P value of <0.05 was considered significant.

RESULTS

Implantation of MKN45c185 and 85As2 cells induced cancer cachexia in rats. Subcutaneous implantation of either MKN45c185 or 85As2 cells in rats led to progressive tumor growth beginning 1 wk after implantation and affected body weight and composition. Body weight was markedly reduced 2 wk after implantation of MKN45c185 and 85As2 cells compared with controls, and thereafter, the differences gradually increased (Fig. 1, A and B). Additionally, all body composition parameters (FFM, FM, and TBW) were significantly lower in the MKN45c185 and 85As2 groups than in the control group (Fig. 1, C–E). Moreover, all muscle and adipose tissue weights

Fig. 3. Atrophy and expression of E3 ubiquitin ligases in the gastrocnemius muscle in 85As2-bearing cachectic rats 4 wk after implantation. Nude rats were inoculated subcutaneously with 85As2 cells (1 × 10⁷ cells/site) or saline (control) in both flanks at week 0. A: macroscopic observation of the gastrocnemius muscle in a control rat (top muscle in the photo) and cachectic rat (bottom muscle in the photo). B and C: histological observation of the gastrocnemius muscle tissue in a control rat (B) and cachectic rat (C). D and E: expression of the E3 ubiquitin ligases atrogin-1 (D) and muscle RING finger 1 (MuRF-1; E) in gastrocnemius muscle tissue. Each bar represents the mean ± SE of 7–8 rats. Differences between groups were evaluated using the Mann-Whitney U-test. *P < 0.05 and ***P < 0.001 vs. the control group.

Fig. 4. Plasma levels of α1-acid glycoprotein (AGP; A) and albumin (B) at 2, 4, and 12 wk after 85As2 cell implantation in nude rats. Rats were inoculated sc with 85As2 cells (1 × 10⁷ cells/site) or saline (control) in both flanks at week 0. Each bar represents the mean ± SE of 5 rats. Differences between groups were evaluated using Student’s t-test or Welch’s t-test. *P < 0.05 and ***P < 0.001 vs. the control group.
were significantly reduced in cachectic rats at 12 wk after implantation compared with control rats (Fig. 1F). Symptoms of cancer cachexia, including weight loss, low FM and FFM, and wasting of muscle and adipose tissues, were more pronounced in the 85As2 group than in the MKN45cl85 group.

85As2-induced cancer cachexia rat model characterization. Because the 85As2 model induced more severe cancer cachexia in rats than the MKN45cl85 model, the 85As2-induced cancer cachexia model was characterized further. Tumor volume grew progressively in a cell concentration-dependent manner, reaching 1.94 ± 0.57 and 4.38 ± 0.68 cm³ at 4 wk after implantation of 1 × 10⁶ and 1 × 10⁷ 85As2 cells, respectively (Fig. 2B). The body weight of the control group continued to increase during the experiment, whereas the body weight of the 85As2 groups did not. Body weight loss was higher in the 85As2 groups than in the control group beginning...
Table 2. Plasma levels of human cytokines in the cancer cachexia rat models and cell culture supernatants

<table>
<thead>
<tr>
<th>Time after Inoculation</th>
<th>Cells</th>
<th>IL-1β</th>
<th>IL-6</th>
<th>IL-8</th>
<th>TNFα</th>
<th>LIF</th>
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<tr>
<td>85As2</td>
<td>$1 \times 10^6$</td>
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Supernatant, 24 h

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<th>IL-1β</th>
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<th>IL-8</th>
<th>TNFα</th>
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<tbody>
<tr>
<td>85As2</td>
<td>$5 \times 10^3$</td>
<td>ND</td>
<td>ND</td>
<td>10.36</td>
<td>611.74</td>
</tr>
<tr>
<td>MKN45cI85</td>
<td>$5 \times 10^3$</td>
<td>ND</td>
<td>ND</td>
<td>321.47</td>
<td>416.00</td>
</tr>
</tbody>
</table>

Supernatant, 48 h

<table>
<thead>
<tr>
<th>Cells</th>
<th>IL-1β</th>
<th>IL-6</th>
<th>IL-8</th>
<th>TNFα</th>
<th>LIF</th>
</tr>
</thead>
<tbody>
<tr>
<td>85As2</td>
<td>$5 \times 10^3$</td>
<td>ND</td>
<td>ND</td>
<td>19.02</td>
<td>937.29</td>
</tr>
<tr>
<td>MKN45cI85</td>
<td>$5 \times 10^3$</td>
<td>ND</td>
<td>ND</td>
<td>359.98</td>
<td>724.91</td>
</tr>
</tbody>
</table>

Cytokine levels in cell culture supernatants are expressed as the mean ± SE of triplicate wells in pg/ml, and plasma cytokine levels are expressed as the mean ± SE (pg/ml) values for 4–5 rats. LIF, leukemia inhibitory factor; ND, not detectable (below the minimum detection limit of the assay). Rats were implanted subcutaneously with MKN45cI85 or 85As2 cells ($1 \times 10^5$ or $10^7$ cells/site) or saline alone in both flanks. Differences in plasma cytokine levels between groups were evaluated using the Kruskal-Wallis test, followed by a post hoc Dunn’s multiple comparison test ($*P < 0.05$ and $**P < 0.01$ vs. the corresponding control group). Supernatants were collected from 24- or 48-h incubation cultures. Differences in the cytokine levels in cell culture supernatants for the groups were evaluated using Student’s $t$-test ($**P < 0.01$ and $***P < 0.001$ vs. the corresponding MKN45cI85 group).

at 2 wk after implantation and became significant at 3 and 2 wk after implantation of $1 \times 10^5$ and $1 \times 10^7$ cells, respectively (Fig. 2A). The differences in body weight between the 85As2-implanted groups and corresponding control groups were greatest at 4 wk after implantation. The differences in body weight between the 85As2 groups and control group were 70.1–74.3 and 82.7–84.7% regardless of body weight with or without the tumor weight at 4 wk after implantation of $1 \times 10^7$ cells, respectively. Determination of the mRNA levels in the brain regions (Fig. 2B and C) showed significant decreases in the expression of the neuropeptide Y (NPY), agouti-related protein (AgRP), proopiomelanocortin (POMC), and cocaine- and amphetamine-regulated transcript (CART) in the arcuate nucleus (ARC), corticotropin-releasing hormone (CRH) in the paraventricular nucleus (PVN), and orexin (OXR) and melanin-concentrating hormone (MCH) in the lateral hypothalamic area (LHA) in control and 85As2-induced cachectic rats 4 wk after implantation. Nude rats were inoculated subcutaneously with 85As2 cells ($1 \times 10^5$ cells/site) or saline (control) in both flanks at week 0. In situ hybridization was measured 4 wk after implantation. Representative autoradiographs of sections hybridized by 35S-labeled oligodeoxynucleotide probe complementary to mRNA for all the peptides mentioned in A. Signal intensity ranges from high (black bars) to low (open bars). Black bar = 1 mm. B: $t$-test for changes in body weight (B) and food intake (C). Changes in body weight and food intake were evident at 4 wk after implantation. Each bar or data point represents the mean ± SE of 6 rats. Differences between groups were evaluated using Student’s $t$-test. $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$ vs. the control group.
and 1 × 10^6 cells, respectively (Table 1). Comparison between peak body weight and body weight 4 wk after implantation showed body weight loss in each cachectic rat (1 × 10^7 cells, 88.0 ± 3.5%; and 1 × 10^6 cells, 94.4 ± 2.7%). These comparisons were made using the corresponding individual peak body weight. All body composition parameters (FFM, FM, and TBW) were also substantially lower in the 85As2 groups than in the control groups (Fig. 2, E–G).

Reductions in food and water intake were observed beginning at 1 wk after implantation in the 85As2 groups compared with the corresponding control groups and became significant 2–4 wk later (food intake: 1 × 10^7 cells, 73.5–78.7%; and 1 × 10^6 cells, 89.0–90.7%; water intake: 1 × 10^7 cells, 71.2–80.1%; and 1 × 10^6 cells, 83.4–88.7%; Fig. 2, C and D). Similarly to the 85As2-induced cachexia model, MKN45cl85-implanted rats also exhibited marked decreases in food intake (Fig. 2H). However, the decrease in food intake was less pronounced in MKN45cl85-implanted rats than in 85As2-implanted rats (MKN45cl85, 1 × 10^7 cells, 83.8–87.0% compared with the corresponding control groups).

Muscle (greater pectoral, gastrocnemius, tibialis, and soleus), adipose tissue (epididymal, perirenal, and mesentery fat), liver, and spleen weights decreased substantially in a cell concentration-dependent manner at 4 wk after implantation in cachectic rats compared with that in control rats (Table 1). Macroscopic and histological observations confirmed gastrocnemius muscle atrophy in the 85As2 group (1 × 10^7 cells) at 4 wk after implantation compared with that in control rats (Table 1). Macroscopic and histological observations confirmed gastrocnemius muscle atrophy in the 85As2 group (1 × 10^7 cells) at 4 wk after implantation (Fig. 3, A–C). Furthermore, all cells in Fig. 2, B and C (control, n = 51; 85As2, n = 75), were measured, and the cross-sectional area, perimeter, Feret diameter, and minimum Feret diameter were calculated. The cross-sectional area (1,460.4 ± 76.3 vs. 2,023.9 ± 85.2 μm^2, P <
NEW CANCER CACHEXIA MODEL

A

Food intake (g/day)

-14 -13 -12 -11 -10 -9 -8 -7 -6 -5 -4 -3 -2 -1 0 1 2 3 4 5 6 7

Days

-☆☆
-☆☆☆
-☆☆☆
-☆☆☆
-☆☆☆
-☆☆☆

Control + distilled water
85As2 (1x10^7 cells) + distilled water
85As2 (1x10^7 cells) + rikkunshito

B

Water intake (mL/day)

-14 -13 -12 -11 -10 -9 -8 -7 -6 -5 -4 -3 -2 -1 0 1 2 3 4 5 6 7

Days

-☆☆☆
-☆☆☆
-☆☆☆
-☆☆☆
-☆☆☆
-☆☆☆

Control + distilled water
85As2 (1x10^7 cells) + distilled water
85As2 (1x10^7 cells) + rikkunshito

C

Cumulative food intake (g)

0 50 100 150

Days

0 1 2 3 4 5 6 7

-☆☆☆
-☆☆☆
-☆☆☆
-☆☆☆

Control + distilled water
85As2 (1x10^7 cells) + distilled water
85As2 (1x10^7 cells) + rikkunshito

D

Cumulative water intake (mL)

0 50 100 150

Days

0 1 2 3 4 5 6 7

-☆☆☆
-☆☆☆
-☆☆☆
-☆☆☆

Control + distilled water
85As2 (1x10^7 cells) + distilled water
85As2 (1x10^7 cells) + rikkunshito

E

Food intake (g/day)

before administration
after administration

-☆☆☆
-☆☆☆

Control + distilled water
85As2 (1x10^7 cells) + distilled water
85As2 (1x10^7 cells) + rikkunshito

F

Water intake (mL/day)

before administration
after administration

-☆☆☆
-☆☆☆

Control + distilled water
85As2 (1x10^7 cells) + distilled water
85As2 (1x10^7 cells) + rikkunshito

G

Body weight (g)

0 100 200 300 400

Saline
85As2 (1x10^7 cells)
distilled water
rikkunshito

H

FFM (g)

100 150 200

Control
85As2 (1x10^7 cells)
distilled water
rikkunshito

before administration
after administration

I

TWI (liter)

0.01 0.12 0.14

Control
85As2 (1x10^7 cells)
distilled water
rikkunshito

before administration
after administration

J

Weight (g)

0 2 4 6 8

Total Muscle
Total Fat

Control + distilled water
85As2 (1x10^7 cells) + distilled water
85As2 (1x10^7 cells) + rikkunshito
Hypothalamic feeding-regulating peptide levels were evaluated using the paired t-test; *P < 0.05, **P < 0.01, and ***P < 0.001 vs. the corresponding before-administration group (E and G). Differences between groups in the time course of cumulative food intake (A, B, and C) were evaluated using Student’s t-test; *P < 0.05, **P < 0.01, and ***P < 0.001 vs. the corresponding control + distilled water-treated group (A, B, and C). Differences between the LHA, PAR, and the VPL were evaluated using the paired t-test; *P < 0.05, **P < 0.01, and ***P < 0.001 vs. the corresponding before-administration group (E and G). Differences between groups were evaluated using 1-way ANOVA, followed by post hoc Dunnett’s multiple comparison test; **P < 0.01 and ***P < 0.001 vs. the 85As2 + distilled water-treated group (E and F). Differences before and after administration of either rikkunshito or distilled water were evaluated using the paired t-test; #P < 0.05, ##P < 0.01, ###P < 0.001 vs. the corresponding before-administration group (E and G).
atrogin-1 and been associated with muscle wasting in other cancer cachexia. Skeletal muscle loss by accelerating muscle protein breakdown is mediated by ubiquitin ligases atrogin-1 and the proteasome system (2, 16), increased expression of the E3 ubiquitin ligase atrogin-1 and MuRF-1 likely contributed to skeletal muscle loss by accelerating muscle protein breakdown during cancer cachexia development, as these enzymes have been associated with muscle wasting in other cancer cachexia animal models (2, 16). Moreover, increased expression of atrogin-1 and MuRF-1 in the gastrocnemius muscles of cachectic rats has been associated with increased expression of Foxo1, the master regulators of muscle-specific E3 ligases, and increased Foxo expression has been shown in cancer cachexia models (49). In our study, Foxo1, Foxo3, and Foxo4 were upregulated in 85As2-induced cachetic rats, and their increased expression was thought to be associated with the increased expression of atrogin-1 and MuRF-1. Notably, the elevation of Foxo1 levels was prominent, and its blockade suppressed cachetic muscle atrophy (36). Expression of the protein synthetic factor IGF-1 has been reported to decrease in cancer cachexia models (16). However, IGF-1 expression did not decrease in cachetic rats; rather, it unexpectedly increased, although the increase was not significant. Taken together, our findings indicated that protein degradation pathways in skeletal muscle were activated in 85As2-induced cachetic rats. Importantly, tumor removal reversed the cachexia symptoms, including anorexia, in the present study by producing by 85As2 and MKN45c185 cell implantation induced cachexia symptoms, including anorexia, in the present study by affecting LIF receptor signaling pathways in POMC neurons in the ARC. Moreover, LIF may contribute to differences in the onset and severity of cachexia in the 85As2 and MKN45c185 cachexia models. However, further study is necessary to determine the contribution of other cachetic factors to the varying degrees of cancer cachexia in these models.

In the present study, hypothalamic levels of orexigenic peptide mRNAs (NPY and AgRP in the ARC, ORX and MCH in the LHA) were increased in the 85As2-induced cachexia model, whereas the levels of anorexigenic peptide mRNAs (POMC and CART in the ARC, CRH in the PVN) were decreased. Previous studies have shown that hypothalamic NPY release is reduced and that the feeding response to hypothalamic injection of NPY is attenuated in anorectic tumor-bearing rats despite increased hypothalamic NPY mRNA expression (9–12, 38). Moreover, proinflammatory signals (e.g., IL-1β) have been shown to decrease AgRP secretion but increase AgRP gene transcription (50). Thus, despite increases in hypothalamic NPY and AgRP mRNA expression, the anorexia induced in our model may involve impairment of NPY and AgRP release or feeding response to NPY. Interestingly, our previous study using a cisplatin-induced cachexia rat model yielded contrasting results to those of the 85As2 model, although both models exhibited decreased food intake. In the cisplatin-induced cachexia model, hypothalamic orexigenic peptide mRNA levels decreased and anorexigenic peptide mRNA levels increased (65). Cisplatin has been shown to reduce the secretion of ghrelin that activates NPY neurons,
whereas it inhibits POMC and CART expression in the ARC (34, 57). Reduced ghrelin secretion may decrease NPY mRNA levels and increase POMC and CART mRNA levels in the ARC. These findings indicated that distinct underlying mechanisms may induce cachexia-associated anorexia development in different cachexia models.

In addition to body weight loss and anorexia, patients with cancer cachexia also exhibit a reduction in physical activity corresponding to daytime activities (22, 61). Similarly, locomotor activity in the “active period” or dark phase was substantially lower in 85As2-induced cachetic rats than in control rats, whereas locomotor activity was not different between these groups during the daytime period. Reduced activity during the dark phase, but not the light phase, in cachetic rats correlated with decreased food intake (22, 61). In fact, increased resting energy expenditure in patients with cancer cachexia has frequently been observed (7, 8, 21) and is in contrast to the resting energy conservation associated with starvation-induced body weight loss.

Body weight maintenance is the most important end point of any treatment for cachexia-associated anorexia. Rikkunshito therapy has been shown to be an effective anorexia treatment in several animal models (50, 57); therefore, we evaluated the effect of rikkunshito on 85As2-induced cachexia symptoms. Rikkunshito substantially ameliorated cancer cachexia symptoms, including anorexia, weight loss, decreased water intake, and reductions in FFM, TBW, and musculature in the 85As2-induced cancer cachexia rat model; however, rikkunshito did not reduce tumor growth or plasma LIF levels. These findings indicated that the anticachectic effects of rikkunshito are not related to tumor regression or LIF levels. Rikkunshito has been shown to increase the secretion of ghrelin, an orexigenic hormone (57), and also to increase ghrelin receptor (GHSR) signaling efficacy (24). GHSR is expressed in the ARC and PVN of the hypothalamus, and ligand binding stimulates NPY/AgRP neurons, thereby transducing orexigenic signals to increase food intake. Thus, rikkunshito may ameliorate anorexia by activating GHSR-NPY/AgRP orexigenic signaling in the ARC and PVN. In fact, our previous study demonstrated that rikkunshito ameliorated cisplatin-induced anorexia in rats and reversed the cisplatin-induced decrease in hypothalamic orexigenic peptide mRNA levels (NPY in ARC) and increase in anorexigenic peptide mRNA levels (POMC and CART in the ARC) (65). Because anorexia induced by cancer cachexia and cisplatin may involve different mechanisms, further study is required to clarify the mechanisms by which rikkunshito ameliorates cancer cachexia-induced anorexia.

In conclusion, we established novel stomach cancer cachexia rat models by implanting nude rats with MKN45cI85 and 85As2 cells, both of which were derived from the human stomach cancer cell line MKN-45. The 85As2-induced cancer cachexia model, which was generated using peritoneal dissemination-derived 85As2 cells, induced earlier and more severe cachexia than the MKN45cI85 model, which may have been caused by differences in LIF production. The 85As2 model allowed for the early evaluation of cancer cachexia parameters associated with poor patient QOL and metabolic disturbances, such as anorexia and body weight loss (including low FFM). Our findings also indicate that rikkunshito may improve QOL in patients with stomach cancer cachexia. The 85As2 model should provide a useful tool for further study of cancer cachexia pathogenesis and treatment.

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DISCLOSURES

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AUTHOR CONTRIBUTIONS


REFERENCES


