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

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Abstract

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 2 novel cell lines: 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.

Keywords: cachexia, leukemia inhibitory factor, rikkunshito, stomach cancer model, anorexia
Introduction

Cancer cachexia, a multi-factorial 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 non-lymphocytic 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 3 of the following 5 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. reported that 2 specific indicators, anorexia and metabolic disturbances, should be identified before arriving at a diagnosis of cachexia-associated weight loss (1). Although body weight maintenance is the most important endpoint 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). Based on these findings, we established 2 novel cell lines from MKN-45 cells: MKN45cl85 and 85As2 (63).
Implantation of MKN45cl85 cells induces body weight loss in mice with 100% efficiency. The
85As2 cell line, derived from peritoneal metastasis of orthotopically implanted MKN45cl85 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). Therefore, 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 2
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/muscle atrophy F-box (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 rikkunshito increases the secretion of the orexigenic hormone ghrelin through
inhibition of 5-HT2B/2C receptors, enhancement of ghrelin receptor (GHSR) signaling efficacy, and
facilitation of gastric emptying, gastric adaptive relaxation and upper gastrointestinal motility and
may thus help ameliorate anorexia (24, 32, 57, 60, 64). Herein, we specifically examined the efficacy
of rikkunshito in ameliorating anorexia symptoms in our novel cancer cachexia rat models.

Materials and Methods

Animal experimentation

Six-week-old male F344/NJcl-rnu/rnu rats (Clea-Japan, Tokyo, Japan) were housed
individually under a 12:12-h light-dark cycle (lights on at 08:00 AM) at a constant temperature and
humidity with ad libitum access to food and water. Rats were allowed to acclimate to laboratory
conditions for 2 weeks prior to experimentation. All studies were performed according to the
Guidelines for Animal Experiments drafted by the Committee for Ethics in Animal Experimentation
of the National Cancer Center and met the ethical standards required by the law and the guidelines
concerning experimental animals in Japan.

Cell lines and culture conditions

MKN45c185 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,
Inc, Kyoto, Japan) supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA, USA),
100 IU/mL penicillin G sodium, and 100 μg/mL streptomycin sulfate (Nacalai Tesque, Inc) under a
5% CO₂ and 95% air atmosphere at 37°C.

Tumor cell implantation

MKN45c185 and 85As2 cells were harvested from subconfluent cultures after brief exposure to
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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 $1 \times 10^6$–$10^7$ 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 ($\text{cm}^3$) = major axis (cm) $\times$ minor axis (cm) $\times$ minor axis (cm) $\times$ 1/2, and tumor volume was converted to tumor weight (mg)/mm$^3$ (14, 17, 66).

**Rat model characterization**

Body weight and composition, organ tissue weight, food and water consumption, and grip strength were evaluated in each model. Body weight and food and water consumption were measured weekly. Body composition was determined using the ImpediVET™ Bioimpedance Spectroscopy device (ImpediMed Limited, Brisbane, Australia) to accurately measure FFM, fat mass (FM), and total body water (TBW) (52). Blood collected from the abdominal aorta was centrifuged (3000 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 ImageJ software (15). Grip strength was measured using a grip-strength meter for rats and mice (MK-380CM/F; Muromachi Kikai Co., Ltd, Tokyo, Japan).

**Tumor removal experiment**

Anesthetized rats were subcutaneously inoculated with either 85As2 $1 \times 10^7$ cells/site (tumor-bearing rats) or saline alone (non-tumor-bearing control rats) in the left and right flanks. Tumor growth, body weight, food and water intake were measured weekly. Cancer cachexia clearly developed in tumor-bearing rats 2 weeks after implantation. After that, tumor-bearing cancer cachectic rats were divided into 2 groups: one group was not treated and the other group had their
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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 week 0 (before tumor implantation), week 2 (before tumor removal), and weeks 4 and 5 (after tumor removal). Grip strength was measured, and the plasma and gastrocnemius muscle were collected 5 weeks after implantation.

**Real-time polymerase chain reaction**

Real-time polymerase chain reaction (PCR) was performed as previously described (56). Briefly, the hypothalamic area was dissected on an ice-cold metal plate, and total RNA was isolated using an Isogen kit (Nippon gene Co., Ltd, 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, USA) 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, (forward) 5’-GAA GAC CGG CTA CTG TGG AA-3’ and (reverse) 5’-ATC AAT CGC TTG CGG ATC T-3’; MuRF-1, (forward) 5’-AGG ACT CCT GCC GAG TTA GTG AGC A-3’ and (reverse) 5’-TTG TGG CTC AGT TCC TCC TT-3’; forkhead box O1 (Foxo-1), (forward) 5’-TCA GGC TAG GAG TTA GTG AGC A-3’ and (reverse) 5’-AAG GAG GGG TGA AGG GCA TC-3’; Foxo-3, (forward) 5’-CTC AGC CAG TGG ACA GTG AA-3’ and (reverse) 5’-TCC GAG CCC ATC TGC TTG TTC A-3’; Foxo-4, (forward) 5’-GCC GAG CCT GTC CTT ATC CA-3’ and (reverse) 5’-TTG AGT GTG TGA CCC TTT AGT-3’; IGF-1, (forward) 5’-GCA CTC TGC TTG CTC ACC TTT A-3’ and (reverse) 5’-TCC GGA TGC TGG AGC CAT A-3’ and glyceraldehyde-3-phosphate dehydrogenase (forward) 5’-CCC CCA ATG TAT CCG TTG TG-3’ and (reverse) 5’-TAG CCC AGG ATG CCC TTT AGT-3’. PCR products were quantified using LightCycler 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 (C$_T$) using the $2^{-\Delta\Delta C_T}$ method (37).

**Cytokine measurements**

Plasma levels of human IL-1β, IL-6, IL-8, TNF-α, and LIF were measured using the Luminex Multiplex Assay (Affymetrix Billerica, MA, USA) (18). Rat IL-1β, IL-6, TNF-α, keratinocyte-derived chemokine (KC), and interferon (IFN)-γ plasma levels were measured using the Procarta® Cytokine Assay Kit (Affymetrix Billerica, MA, USA). Plasma α1-acid glycoprotein and albumin levels were measured using a rat α1-acid glycoprotein enzyme-linked immunosorbent assay (ELISA) kit (Immunology Consultants Laboratory, Inc., Newberg, OR, USA) and a rat albumin ELISA kit (Shibayagi, Gunma, Japan), respectively. Human cytokine levels were also measured in MKN45cl85 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 previously described (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 (PVN), arcuate nucleus (ARC), and lateral hypothalamic area (LHA) were identified according to the Paxinos and Watson atlas (48) and confirmed by microscopy. Hybridization was conducted under a Nescofilm coverslip (Bando Chemical IMD, Osaka, Japan). [35S]3′-end-labeled deoxyoligonucleotides complementary to transcripts coding for neuropeptide Y (NPY) (5′-GGA GTA GTA TCT GGC CAT GTC CTC TGC TGG CGC GTC-3′), agouti-related protein (AgRP) (5′-CGA CGC GGA GAA CGA GAC TCG CGG TTC TGT GGA TCT AGC ACC TCT GCC-3′), proopiomelanocortin (POMC) (5′-CTT CTT GCC CAG CGG CTT GCC CCA GCA GAA GTG CTC CAT GGA CTA GGA-3′), cocaine- and amphetamine-regulated transcript (CART) (5′-TGG GGA CTT GGC CGT ACT TCT TCT CAT AGA TCG GAA TGC-3′), orexin (5′-TTC GTA GAG ACG GCA GGA ACA CGT CTT CTG GCG ACA-3′), corticotropin-releasing hormone (CRH) (5′-CAG TTT CCT GTT GCT GTG AGC TTG CTG AGC TAA CTG CTC TGC CCT GGC-3′), and melanin-concentrating hormone.
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(MCH) (5’-CCA ACA GGG TCG GTA GAC TCG TCC CAG CAT-3’) were used as gene-specific probes (28, 30, 31, 43, 62). Total counts of $6 \times 10^5$ cpm/slide for NPY, AgPR, POMC, CART, MCH, and CRH and $4 \times 10^5$ cpm/slide for orexin were used. Hybridized sections containing the ARC, LHA, and PVN regions were exposed to autoradiography film (Hyperfilm; Amersham, Buckinghamshire, UK) for 4 days for orexin and 7 days for NYP, AgRP, POMC, CART, MCH, and CRH. Autoradiographic images were captured at $40\times$ magnification and quantified using an MCID imaging analyzer (Imaging Research, St. Catherines, ON, Canada). The images were captured by a charge-coupled device camera (Dage-MTI, Michigan City, IN). Mean absorbance was measured and compared with simultaneously exposed $^{14}$C microscale samples (Amersham). The standard curve was fitted according to the absorbance of the $^{14}$C microscale on the same film.

**Respiratory metabolism**

Oxygen consumption was measured with an O$_2$/CO$_2$ metabolism-measuring system (MK-5000RQ; Muromachi Kikai Co., Ltd, 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 (VO$_2$) and carbon dioxide production (VCO$_2$) were calculated (mL·min$^{-1}$·kg$^{-1}$). Locomotor activity was measured simultaneously with an attached device. The respiration quotient (RQ) was calculated by dividing VCO$_2$ by VO$_2$. Metabolic calories (E) were calculated using the system software as follows: $E$ (cal·min$^{-1}$·kg$^{-1}$) = $(1.07 \times RQ + 3.98) \times VO_2/\text{body weight}$.

**Palliative therapeutic studies using rikkunshito**

Rikkunshito was manufactured by Tsumura and Co. (Tokyo, Japan) by spray-drying a hot water extract from the following 8 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), *Glycyrrhizae radix* (1.0 g), and *Zingiberis rhizoma* (0.5 g). The powdered rikkunshito extract was obtained from Tsumura and Co. For oral administration into the
stomach using a disposable sonde, rikkunshito was dissolved in distilled water (DW) at g/10 mL in our laboratory. Rats were implanted s.c. with 85As2 cells in both flanks (1 × 10^7 cells/each site) on day -14. Rats inoculated with saline served as the non-tumor bearing control group. Tumor-bearing rats were divided into 2 groups: treatment (85As2 + rikkunshito) group and tumor-bearing control (85As2 + distilled water) group. The treatment group was orally administered rikkunshito twice daily at 1000 mg·kg⁻¹day⁻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 + distilled water 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 day -14 (before tumor implantation), day 0 (before administration), and day 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 the mean ± standard error of the mean (SEM). 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 Dunnett’s multiple comparison test, or Kruskal-Wallis test followed by a post-hoc Dunn’s multiple comparison test. A p value < 0.05 was considered significant.

**Results**

*Implantation of MKN45cl85 and 85As2 cells induced cancer cachexia in rats*

Subcutaneous implantation of either MKN45cl85 or 85As2 cells in rats induced progressive tumor growth beginning 1 week after implantation and affected body weight and composition. Body
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weight was markedly reduced 2 weeks after implantation of MKN45cl85 and 85As2 cells compared with controls, and thereafter, the differences gradually increased (Figures 1A-B). Additionally, all body composition parameters (FFM, FM, and TBW) were significantly lower in the MKN45cl85 and 85As2 groups than in the control group (Figures 1C-E). Moreover, all muscle and adipose tissue weights were significantly reduced in cachectic rats at 12 weeks after implantation compared with control rats (Figure 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 further characterized. Tumor volume grew progressively in a cell concentration-dependent manner, reaching 1.94 ± 0.57 cm$^3$ and 4.38 ± 0.68 cm$^3$ at 4 weeks after implantation of $1 \times 10^6$ and $1 \times 10^7$ 85As2 cells, respectively (Figure 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 at 2 weeks after implantation and became significant at 3 and 2 weeks after implantation of $1 \times 10^6$ and $1 \times 10^7$ cells, respectively (Figure 2A). The differences in body weight between the 85As2-implanted groups and corresponding control groups were greatest at 4 weeks 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 weeks after implantation of $1 \times 10^7$ cells and $1 \times 10^6$ cells, respectively (Table 1). Comparison between peak body weight and body weight 4 weeks after implantation showed body weight loss in each cachectic rat ($1 \times 10^7$ cells, 88.0% ± 3.5% and $1 \times 10^6$ 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 (Figures 2E-G).
Reductions in food and water intake were observed beginning at 1 week after implantation in the 85As2 groups compared with the corresponding control groups and became significant 2–4 weeks later (food intake: $1 \times 10^7$ cells, 73.5–78.7% and $1 \times 10^6$ cells, 89.0–90.7%; water intake: $1 \times 10^7$ cells, 71.2–80.1% and $1 \times 10^6$ cells, 83.4–88.7%) (Figures 2C-D). Similar to the 85As2-induced cachexia model, MKN45cl85-implanted rats also exhibited marked decreases in food intake (Figure 2H). However, the decrease in food intake was less pronounced in MKN45cl85-implanted rats than in 85As2-implanted rats (MKN45cl85, $1 \times 10^7$ 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 substantially decreased in a cell concentration-dependent manner at 4 weeks 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 ($1 \times 10^7$ cells) at 4 weeks after implantation (Figures 3A-C). Furthermore, all cells in Figures 3B and 3C (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 ($1460.4 \pm 76.3 \mu m^2$ vs. $2023.9 \pm 85.2 \mu m^2$; $p < 0.0001$), perimeter ($151.1 \pm 4.3 \mu m$ vs. $179.2 \pm 4.1 \mu m$; $p < 0.0001$), Feret diameter ($57.5 \pm 1.7 \mu m$ vs. $67.2 \pm 1.8 \mu m$; $p < 0.001$), and minimum Feret diameter ($36.4 \pm 1.1$ vs. $44.1 \pm 1.2 \mu m$; $p < 0.0001$) were lower in the 85As2 group than in the control group. A corresponding increase in the expression of muscle-specific E3 ubiquitin ligases (atrogin-1 and MuRF-1; Figures 3D-E) and reduction in grip strength were also observed (Figure 5K). Three Foxo family members present in skeletal muscle (Foxo-1, Foxo-3, and Foxo-4) (49) were upregulated in the gastrocnemius muscles of the 85As2-induced cachectic rats, and the increase in Foxo-1 was particularly prominent (Figures 5G-I). IGF-1 was upregulated in cachectic rats (non-significant) (Figure 5J). Plasma albumin level decreased in 85As2 ($1 \times 10^7$ cells)-bearing cachectic rats, whereas the level of $\alpha 1$-acid glycoprotein, the murine counterpart of human C-reactive protein, was increased (Figures 4A-B). Importantly, tumor removal restored body weight loss, food and water intake, body composition (FFM, FM, and TBW), and grip strength (Figures
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Furthermore, tumor removal reduced the increased expression levels of not only atrogin-1 (85As2, 321.2% ± 123.2%; tumor removal, 155.2% ± 40.8%) and MuRF-1 (85As2, 261.5% ± 71.3%; tumor removal, 150.7% ± 27.0%) but also Foxo-1 (85As2, 683.7% ± 368.7%; tumor removal, 140.5% ± 29.6%) in the gastrocnemius muscle (Figures 5G-I).

Cytokine levels

To investigate the underlying causes of cancer cachexia, the plasma concentrations of several proinflammatory cytokines were measured. Human LIF levels were remarkably elevated in a cell concentration- and time-dependent manner in rats implanted with 85As2 cells, whereas the levels of human IL-1β, IL-6, and TNF-α were not elevated in this model at 12 weeks (Table 2). Similar results were obtained in MKN45cl85-implanted rats at 12 weeks. Additionally, rat IL-1β, IL-6, KC, and TNF-α levels were below detection limits, and IFN-γ levels were unchanged in both rat models (data not shown). Moreover, human LIF production was observed in cell culture supernatants from both cell lines, although 85As2 cells produced substantially higher amounts of LIF than MKN45cl85 cells (Table 2). Human IL-8 production was also observed in both cell lines. Furthermore, tumor removal reversed the increase in plasma LIF levels in 85As2-bearing cachectic rats (Figure 5L).

Gene expression of hypothalamic orexigenic/anorexigenic peptides in the 85As2-induced cachexia model

Cachexia symptoms such as body weight loss and anorexia were induced in rats implanted with 85As2 cells (1 × 10^7 cells) (Figures 6B-C). Hypothalamic feeding-regulating peptide levels were evaluated 4 weeks after implantation of 85As2 cells. Orexigenic peptide mRNA levels (NPY and AgRP in the ARC; ORX and MCH in the LHA) were higher in 85As2 cachectic rats than in control rats, whereas anorexigenic peptide mRNA levels (POMC and CART in the ARC; CRH in the PVN) were lower in cachectic rats than in control rats (Figure 6A).

Respiratory metabolism in the 85As2-induced cachexia model
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Cachexia symptoms such as body weight loss and anorexia were induced in rats implanted with 85As2 cells (1 × 10^7 cells) (Figures 7G-H). VO₂ was higher in 85As2-induced cachectic rats than in control rats at 4 weeks after implantation (Figure 7A). Locomotor activity was noticeably lower during the “active” overnight period in cachectic rats than in control rats (Figure 7B and D). RQ, VO₂, and metabolic calorie levels were significantly higher in cachectic rats than in control rats during the daytime period, even though locomotor activity was not different between the groups during this time period (Figures 7C-F).

Rikkunshito ameliorates cachexia in the 85As2-induced cachexia model

Rikkunshito increased food and water intake rates (Figures 8A-F) and alleviated body weight loss, FFM, TBW, and total musculature weight loss in 85As2-induced cachectic rats (Figures 8G-J).

Discussion

Herein, we established novel stomach cancer cachexia models by implanting nude rats with MKN45cl85 and 85As2 cells, both of which were derived from the human stomach cancer cell line MKN-45. These models enabled us to sequentially evaluate anorexia and body composition changes (low FFM) that correspond to poor QOL in human cancer patients. In addition to anorexia and low FFM, the cachexia models showed significant weight loss, reduced musculature and muscle strength, and abnormal biochemistry (increased inflammatory marker levels and low serum albumin levels), thereby fulfilling the cachexia diagnostic criteria (20). Interestingly, cancer cachexia developed earlier and was more severe in the 85As2-bearing model than in the MKN45cl85-bearing model, indicating that 85As2 cells derived from peritoneal dissemination possessed an enhanced ability to cause cachexia. Indeed, the presence of peritoneal metastasis promotes cachexia and is associated with a poor prognosis and low QOL in patients with advanced-stage stomach cancer. Our 85As2-bearing model may provide a useful tool for further study into the mechanisms and potential treatment of cancer cachexia.

Characterization of the 85As2-induced cachexia rat model showed marked weight loss and
reductions in food and water intake. Furthermore, 85As2-induced cachexia decreased FFM, FM, and TBW, which was confirmed by histological observations. In the present experimental models, tumor growth or cachexia developed very early; however, young rats (age, 8 weeks at the time of implantation) were used; therefore, senescence, which is common in human cancer, may have been a limitation of the study. Although 85As2 cell implantation can cause peritoneal dissemination accompanied by ascites (63), the decreased food intake in our model was not associated with either condition. Reduced FFM in cachectic rats was thought to be primarily caused by wasting skeletal muscle and organ tissues, as evidenced by the reduction in all measured musculature weights, muscle atrophy (e.g., gastrocnemius muscle), and reduced spleen and liver weights. Because intracellular skeletal muscle proteins are primarily degraded by the ubiquitin-proteasome system (2, 16), increased expression of the E3 ubiquitin ligases 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 Foxo, the master regulators of muscle-specific E3 ligases, and increased Foxo expression has been shown in cancer cachexia models (49). In our study, Foxo-1, Foxo-3, and Foxo-4 were upregulated in 85As2-induced cachectic rats, and their increased expression was thought to be associated with the increased expression of atrogin-1 and MuRF-1. Notably, the elevation of Foxo-1 levels was prominent, and its blockade suppressed cachectic 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 cachectic 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 cachectic rats. Importantly, tumor removal reversed the cachexia symptoms, including body weight loss, decreased food and water intake, body composition changes, and increased expression of genes that accelerate muscle protein breakdown such as atrogin-1, MuRF-1, and Foxo-1 in 85As2-bearing cachectic rats. These findings strongly indicated that the 85As2 cancer
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cell xenograft induced cachexia symptoms.

Increasing evidence suggests that proinflammatory cytokines, including TNF-α, IL-1, IL-6, IL-10, and TGF-β, may be involved in the development of cancer cachexia (13, 19, 58). For example, high IL-6 levels have been associated with increased inflammation (20) and weight loss in patients with non-small-cell lung, pancreatic, and prostate cancers (19, 46, 51). However, other studies have suggested that cancer cachexia is not fully attributable to IL-6 levels (53). In the present study, human and rat IL-6 were not detected in the plasma of MKN45c185- or 85As2-tumor–bearing cachectic rats or in cell culture supernatants, making it unlikely that IL-6 was a causative factor for cancer cachexia in our experimental model. Moreover, human and rat IL-1β and TNF-α were not detected in the plasma of tumor-bearing cachectic rats, and human IL-10 and TGF-β were not detected in the cell culture supernatants, which was similar to our previous results in a stomach cancer cachexia mouse model (63). In contrast, plasma levels of human LIF, a pleiotropic cytokine belonging to the IL-6 family, were markedly elevated in a cell concentration- and time-dependent manner in rats implanted with 85As2 cells. These findings are in agreement with a previous study showing higher LIF levels in a melanoma SEKI-induced cancer cachexia mouse model (39, 40). Furthermore, we found that tumor removal not only abolished the cachexia symptoms induced by 85As2 cells but also decreased plasma LIF levels to below detectable levels. Therefore, our findings strongly suggested that LIF is a cachectic factor in the 85As2-bearing cachexia model. To date, genetic polymorphisms of cytokines such as IL-1β, IL-8, and IL-10 have been implicated in cachexia pathogenesis in stomach cancer patients (5, 26, 54). Our study is the first to associate LIF with stomach cancer cachexia. Although clinical evaluation of LIF is currently ongoing, LIF may be a biomarker of pathogenesis and a therapeutic target for peritoneal dissemination and cachexia in stomach cancer.

LIF and its receptor LIF-R, a heterodimeric receptor complex consisting of the ligand-specific LIF-R and signal-transducing gp130 subunit (3), are expressed in POMC neurons in the ARC and have been shown to impact signaling in the hypothalamus. LIF has been shown to inhibit food intake by directly activating POMC neurons in the ARC and stimulating the release of alpha-melanocyte-
stimulating hormone, which in turn transduces anorexigenic signals (27). Importantly, the blood-
brain barrier (BBB) is relatively permissive in the ARC, allowing the neurons to access circulating
macromolecules. In fact, Pan et al. showed that peripherally administered LIF reached the brain and
spinal cord by crossing the BBB (47). Taken together, these findings suggested that LIF produced 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 cachectic 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. Similar findings were reported in a cisplatin-induced cachexia model (65). 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 mouse model yielded contrasting results to
those of the 85As2 model even though 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, an
orexigenic hormone, and activate 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
could 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 cachectic 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 cachectic rats has also been observed in other cachexia models (41, 59). Because VO\textsubscript{2} is thought to be affected by the amount of locomotor activity, we evaluated this parameter during the daytime period. VO\textsubscript{2}, RQ, and metabolic calorie levels were significantly higher in cachectic rats than in control rats during this time period. These findings suggested that enhanced energy expenditure, in addition to anorexia, may exacerbate body weight loss caused by the decrease in adipose and muscle tissues in cachectic rats. In fact, exacerbated 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 endpoint 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 anti-cachectic 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 MKN45c185 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 MKN45c185 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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Author contributions

Kiyoshi Terawaki: designed throughout the study, performed most of the experiments and statistical analysis, and drafted the manuscript.

Yumi Sawada, Yohei Kashiwase, Hirofumi Hashimoto, Mitsuhiro Yoshimura, Masami Suzuki, Kanako Miyano, and Yuka Sudo: designed and performed a part of the experiments, performed statistical analysis and ensured quality results.

Kazuyoshi Yanagihara: established cell line used, and designed the study.

Seiji Shiraishi, Yoshikazu Higami, Yoichi Ueta and Yasuhito Uezono: critically assessed the manuscript and provided insightful suggestions for improvement of the manuscript.

Disclosures

The authors have no conflicts of interest to disclose.
References


messenger RNA is increased in medial hypothalamus of anorectic tumor-bearing rats. 


23. **Fujitsuka N, Asakawa A, Hayashi M, Sameshima M, Amitani H, Kojima S,
New cancer cachexia model


New cancer cachexia model


New cancer cachexia model


New cancer cachexia model


New cancer cachexia model

**Figure legends**

Fig. 1 Effects of MKN45cl85 and 85As2 cell implantation on (A) body weight, (B) tumor size, (C) fat-free mass (FFM), (D) fat mass (FM), (E) total body water (TBW), and (F) muscle and adipose tissue weights in nude rats. Rats were inoculated subcutaneously (s.c.) with MKN45cl85 or 85As2 cells in both flanks ($1 \times 10^7$ cells/each site) on week 0. Rats inoculated with saline served as a control group. Each data point represents the mean ± SEM of 4–5 rats. Differences between groups were evaluated using Student’s *t*-test or Welch’s *t*-test. *p < 0.05, **p < 0.01, ***p < 0.001 vs. the control group. Gre: greater pectoral muscle; Gas: gastrocnemius muscle; Sol: soleus muscle; Epi: epididymal fat; Ren: perirenal fat; and Mes: mesentery fat.

Fig. 2 Effects of 85As2 cell implantation at different concentrations on (A) body weight, (B) tumor volume, (C) food intake, (D) water intake, (E) fat-free mass (FFM), (F) fat mass (FM), and (G) total body water (TBW) in nude rats. Rats were inoculated subcutaneously (s.c.) with 85As2 cells ($1 \times 10^6$ or $1 \times 10^7$ cells/each site) or saline (control) in both flanks on week 0. Each data point represents the mean ± SEM of 5–10 rats (0–2W: 10 rats; 3–4W: 5 rats). (H) Food intake comparisons between the MKN45cl85 group and 85As2 groups up to 4 weeks after implantation. Rats were inoculated s.c. with MKN45cl85 or 85As2 cells in both flanks ($1 \times 10^7$ cells/each site) at week 0. Rats inoculated with saline served as a control group. Each data point represents the mean ± SEM of 9–10 rats. Differences between groups were evaluated using Student’s *t*-test or Welch’s *t*-test. *p < 0.05, **p < 0.01, ***p < 0.001 vs. the control group.

Fig. 3 Atrophy and expression of E3 ubiquitin ligases in the gastrocnemius muscle in 85As2-bearing cachectic rats 4 weeks after implantation. Nude rats were inoculated subcutaneously with 85As2 cells ($1 \times 10^7$ cells/each site) or saline (control) in both flanks on week 0. (A) Macroscopic observation of the gastrocnemius muscle in a control rat (upper panel) and cachectic rat (lower panel). Histological observation of the gastrocnemius muscle tissue in a (B) control rat and (C) cachectic rat. Expression of the E3 ubiquitin ligases (D) atrogin-1 and (E) muscle RING finger 1.
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(MuRF-1) in gastrocnemius muscle tissue. Each column represents the mean ± SEM of 7–8 rats. Differences between groups were evaluated using the Mann-Whitney U-test. *p < 0.05, ***p < 0.001 vs. the control group.

Fig. 4 Plasma levels of (A) α1-acid glycoprotein and (B) albumin at 2, 4, and 12 weeks after 85As2 cell implantation in nude rats. Rats were inoculated subcutaneously with 85As2 cells (1 × 10^7 cells/each site) or saline (control) in both flanks on week 0. Each column represents the mean ± SEM of 5 rats. Differences between groups were evaluated using Student’s t-test or Welch’s t-test. *p < 0.05, **p < 0.01, ***p < 0.001 vs. the control group.

Fig. 5 Effects of tumor removal on (A) body weight, (B) food intake, (C) water intake, (D) fat-free mass (FFM), (E) fat mass (FM), (F) total body water (TBW), (G) atrogin-1, (H) muscle RING finger 1 (MuRF-1), (I) forkhead box O (Foxo), (J) insulin-like growth factor (IGF)-1, (K) grip strength, and (L) plasma leukemia inhibitory factor (LIF) levels in nude rats implanted with 85As2 cells (1 × 10^7 cells/each site) or saline (control) in both flanks on week 0. Plasma and the gastrocnemius muscle of the rats were collected 5 weeks after implantation. Each data point or column represents the mean ± SEM of 8–9 rats (A-C, K and L). Each data point or column represents the mean ± SEM of 5 rats (D-J). Differences between groups were evaluated using Student’s t-test or the Mann-Whitney U-test. Differences in plasma LIF levels between groups were evaluated using the Kruskal-Wallis test followed by post-hoc Dunn’s multiple comparison test. *p < 0.05, **p < 0.01, ***p < 0.001 vs. the control group. #p < 0.05, ##p < 0.01, ###p < 0.001 vs. the corresponding 85As2 group.

Fig. 6 mRNA expression of (A) 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 (ORX) and melanin-concentrating hormone (MCH) in the lateral hypothalamic area (LHA) in control and 85As2-induced cachectic rats 4 weeks after implantation. Nude rats were
inoculated subcutaneously with 85As2 cells (1 × 10^7 cells/each site) or saline (control) in both flanks on week 0. *In situ* hybridization was measured 4 weeks after implantation. Representative autoradiographs of sections hybridized by a 35S-labeled oligodeoxynucleotide probe complementary to mRNA for all the peptides mentioned in (A). Signal intensity ranges from high (black) to low (white). Black bar = 1 mm. Time course changes in (B) body weight and (C) food intake. Changes in body weight and food intake were evident at 4 weeks after implantation. Each column or data point represents the mean ± SEM of 6 rats. Differences between groups were evaluated using Student’s *t*-test. *p < 0.05, **p < 0.01 vs. the control group.

Fig. 7 Time course changes in (A) oxygen consumption (VO₂; mL·min⁻¹·kg⁻¹), (B) locomotor activity, (C) respiration quotient (RQ), (D) average cumulative locomotor activity, (E) VO₂ (mL·min⁻¹·kg⁻¹), and (F) metabolic calories (cal·min⁻¹·kg⁻¹) from 10:00 to 14:00 at 4 weeks after 85As2 cell implantation in nude rats. Rats were inoculated subcutaneously with 85As2 cells (1 × 10^7 cells/each site) or saline (control) in both flanks on week 0. Respiratory metabolism was measured 4 weeks after implantation. Changes in (G) body weight and (H) food intake over time. Changes in body weight and food intake were evident at 4 weeks after implantation. Each data or column represents the mean ± SEM of 7 rats. Differences between groups were evaluated using Student’s *t*-test or the Mann-Whitney U-test. *p < 0.05, **p < 0.01, ***p < 0.001 vs. the control group.

Fig. 8 Effects of rikkunshito on anorexia and body composition changes in the 85As2-induced cancer cachexia model. Changes in (A) food intake, (B) water intake, (C) cumulative food intake and (D) cumulative water intake over time. Comparison of (E) food intake, (F) water intake, (G) body weight, (H) fat-free mass (FFM), (I) total body water (TBW), and (J) total muscle and fat weight before and after rikkunshito administration. Rats were implanted subcutaneously with 85As2 cells in both flanks (1 × 10^7 cells/each site) on day -14. Rikkunshito (1g·kg⁻¹·day⁻¹) or distilled water was administered orally twice a day for 7 days from day 0. Rats inoculated with saline served as a control group and were administered distilled water similarly. Each data point or column represents the
mean ± SEM of 10–11 rats. Differences between saline-implanted and 85As2-implanted rats were evaluated using Student’s *t*-test; *p < 0.05, **p < 0.01, ***p < 0.001 vs. the corresponding control + distilled water-treated group (A, B, G-J). Differences between rikkunshito and distilled water treatments were evaluated using Student’s *t*-test; #p < 0.05, ##p < 0.01 vs. the corresponding 85As2 + distilled water-treated group (A, B, H-J). Differences between groups in the time course of cumulative food and water intake were evaluated using two-way repeated measures ANOVA followed by post-hoc Bonferroni test; **p < 0.01, ***p < 0.001 vs. the corresponding control + distilled water-treated group, #p < 0.05, ##p < 0.01, ###p < 0.001 vs. the corresponding 85As2 + distilled water-treated group (C, D). Differences between groups were evaluated using a one-way ANOVA followed by post-hoc Dunnett’s multiple comparison test; **p < 0.01, ***p < 0.001 vs. the 85As2 + distilled water-treated group (E, 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, G).
### Table 1: Body, tumor, muscle, fat, and organ weights in nude rats 4 weeks after implantation with different 85As2 cell concentrations

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>$1 \times 10^6$</th>
<th>$1 \times 10^7$</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Tumor weight (T.W.)</strong></td>
<td>0.00 ± 0.00</td>
<td>5.19 ± 1.54*</td>
<td>11.00 ± 2.31***,#</td>
</tr>
<tr>
<td><strong>Tumor volume (T.V.)</strong></td>
<td>0.00 ± 0.00</td>
<td>3.77 ± 1.27**</td>
<td>8.75 ± 1.50***,##</td>
</tr>
<tr>
<td>(= converted tumor weight; C.T.W.)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Body weight (B.W.)</strong></td>
<td>259.62 ± 5.84</td>
<td>219.78 ± 8.98**</td>
<td>192.96 ± 8.62***</td>
</tr>
<tr>
<td>% of control</td>
<td>100.0 ± 2.3</td>
<td>84.7 ± 3.5***</td>
<td>74.3 ± 3.3***</td>
</tr>
<tr>
<td><strong>B.W. – C.T.W.</strong></td>
<td>259.62 ± 5.84</td>
<td>216.01 ± 10.18**</td>
<td>184.20 ± 9.25***,#</td>
</tr>
<tr>
<td>% of control</td>
<td>100.0 ± 2.3</td>
<td>83.2 ± 3.9***</td>
<td>71.0 ± 3.6***,##</td>
</tr>
<tr>
<td><strong>B.W. – T.W.</strong></td>
<td>259.62 ± 5.84</td>
<td>214.59 ± 10.39***</td>
<td>181.96 ± 9.91***</td>
</tr>
<tr>
<td>% of control</td>
<td>100.0 ± 2.3</td>
<td>82.7 ± 4.0***</td>
<td>71.0 ± 3.6*** ,#</td>
</tr>
<tr>
<td><strong>Peak body weight</strong></td>
<td>226.89 ± 6.06</td>
<td>206.63 ± 7.15</td>
<td></td>
</tr>
<tr>
<td>% of peak body weight</td>
<td>94.4 ± 2.7</td>
<td>88.0 ± 3.5†</td>
<td></td>
</tr>
<tr>
<td><strong>Muscle weights</strong></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Greater pectoral</td>
<td>2.67 ± 0.11</td>
<td>1.88 ± 0.10***</td>
<td>1.42 ± 0.04***,##</td>
</tr>
<tr>
<td>Gastrocnemius</td>
<td>1.37 ± 0.04</td>
<td>1.17 ± 0.04***</td>
<td>0.97 ± 0.06***,##</td>
</tr>
<tr>
<td>Tibialis</td>
<td>0.54 ± 0.02</td>
<td>0.42 ± 0.03***</td>
<td>0.41 ± 0.04**</td>
</tr>
<tr>
<td>Soleus</td>
<td>0.08 ± 0.01</td>
<td>0.07 ± 0.00*</td>
<td>0.07 ± 0.01*</td>
</tr>
<tr>
<td><strong>Fat weights</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Epididymis</td>
<td>3.78 ± 0.19</td>
<td>2.73 ± 0.36*</td>
<td>1.79 ± 0.30***</td>
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<tr>
<td>Perirenal</td>
<td>2.58 ± 0.28</td>
<td>1.53 ± 0.45</td>
<td>0.64 ± 0.34**</td>
</tr>
<tr>
<td>Mesentery</td>
<td>1.41 ± 0.13</td>
<td>0.95 ± 0.29</td>
<td>0.40 ± 0.18**</td>
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<tr>
<td><strong>Organ weights</strong></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Liver</td>
<td>10.36 ± 0.33</td>
<td>7.69 ± 0.56**</td>
<td>6.69 ± 0.31***</td>
</tr>
<tr>
<td>Spleen</td>
<td>0.63 ± 0.03</td>
<td>0.48 ± 0.02**</td>
<td>0.49 ± 0.03**</td>
</tr>
</tbody>
</table>

Rats were subcutaneously implanted with either 85As2 cells ($1 \times 10^6$ or $1 \times 10^7$ cells/site) or saline.
alone in both flanks. Data are expressed as the mean ± SEM of 5 rats. All weight data are expressed in grams. The tumor volume was estimated using the following equation: Tumor volume (cm$^3$) = major axis (cm) × minor axis (cm) × minor axis (cm) × 1/2, and the tumor volume was converted to tumor weight (mg)/mm$^3$. Tumor weight and volume are expressed as the total for both sites. Values for bilateral tissues represent the mean of those for the 2 unilateral tissues. Body weight comparisons between bilateral tissues represent the mean of those for the 2 unilateral tissues. Body weight comparisons between the control group and 85As2 groups at 4 weeks after implantation showed the following relationship: % of control (%) = body weight of each 85As2 group/body weight of control group × 100. Body weight comparisons between peak body weight and body weight at 4 weeks after implantation in each 85As2 group showed the following relationship: % of peak body weight (%) = body weight (minus tumor weight) at 4 weeks after implantation/peak body weight (minus converted tumor weight) × 100. Differences between groups were evaluated using Student’s $t$-test. Differences in tumor weight and volume between the control group and 85As2 groups were evaluated using the Kruskal-Wallis test followed by a post-hoc Dunn’s multiple comparison test. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ vs. the control group, #$p < 0.05$, ##$p < 0.01$ vs. the 85As2 cells 1 × 10$^6$ cells group, †$p < 0.05$ vs. each peak body weight.
Table 2: Plasma levels of human cytokines in the cancer cachexia rat models and cell culture supernatants

<table>
<thead>
<tr>
<th>Weeks after inoculation</th>
<th>Treatment</th>
<th>cells</th>
<th>IL-1β</th>
<th>IL-6</th>
<th>IL-8</th>
<th>TNF-α</th>
<th>LIF</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Plasma</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>2W</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td></td>
<td>&lt;2.44</td>
<td>&lt;2.44</td>
<td>&lt;2.44</td>
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<tr>
<td></td>
<td>85As2</td>
<td>1 × 10^6</td>
<td>&lt;2.44</td>
<td>&lt;2.44</td>
<td>&lt;2.44</td>
<td>&lt;2.44</td>
<td>5.35 ± 3.49</td>
</tr>
<tr>
<td></td>
<td>85As2</td>
<td>1 × 10^7</td>
<td>&lt;2.44</td>
<td>&lt;2.44</td>
<td>&lt;2.44</td>
<td>&lt;2.44</td>
<td>12.94 ± 2.02*</td>
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<td>&lt;2.44</td>
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<td>&lt;2.44</td>
<td>39.88 ± 25.14**</td>
<td>&lt;2.44</td>
<td>321.18 ± 42.02**</td>
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<td>1 × 10^7</td>
<td>&lt;2.44</td>
<td>&lt;2.44</td>
<td>&lt;2.44</td>
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<td>10.36 ± 0.70***</td>
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<td>559.98 ± 25.16</td>
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<td>724.91 ± 22.50</td>
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Rats were subcutaneously implanted with MKN45cl85 or 85As2 cells (1 × 10^6 or 10^7 cells/site) or saline alone in both flanks. Plasma cytokine levels are expressed as the mean ± SEM (pg/mL) values for 4–5 rats. 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, **p < 0.01 vs. the corresponding control group). Supernatants were collected from 24- or 48-h incubation cultures. Cytokine levels in cell culture supernatants are expressed as the mean ± SEM of triplicate wells in pg/mL. Differences in the cytokine levels in cell culture supernatants for the groups were evaluated using the Student’s t-test (***p < 0.01, ****p < 0.001 vs. the corresponding MKN45cl85 group). IL: interleukin; TNF-α: tumor necrosis factor-α; LIF: leukemia inhibitory factor; N.D.: not detected.
detectable (below the minimum detection limit of the assay).
Fig. 1
Fig. 2

(A) Body weight (g)

(B) Tumor volume (cm³)

(C) Food intake (g/day)

(D) Water intake (mL/day)

(E) FM (g)

(F) FM (g)

(G) TBW (liter)

(H) Food intake / day (g)

- 2 -
Fig. 3
Fig. 4
Fig. 5

(K) Grip strength (% of control) for Control, 85As2 (1 × 10^7 cells) and Tumor removal.

(L) Plasma LIF (pg/mL) for Control, 85As2 (1 × 10^7 cells) and Tumor removal.
Fig. 6
**Fig. 7**

(A) VO2 (mL/min/kg) during the dark phase for Control and 85As2 (1x10^7 cells).

(B) Counts/min for Control and 85As2 (1x10^7 cells) during the dark phase.

(C) RQ for Control and 85As2 (1x10^7 cells) during the dark phase.

(D) Comparison of VO2 (mL/min/kg) between Control and 85As2 (1x10^7 cells) from 21:00-7:00 and 10:00-14:00.

(E) VO2 (mL/min/kg) for Control and 85As2 (1x10^7 cells) from 10:00-14:00.

(F) Cal (cal/min/kg) for Control and 85As2 (1x10^7 cells) from 10:00-14:00.

(G) Body weight (g) over weeks after implantation for Control and 85As2 (1x10^7 cells).

(H) Food intake (g/day) over weeks after implantation for Control and 85As2 (1x10^7 cells).
Fig. 8