therapeutic interventions less effective and is responsible for at least 20% of deaths in cancer patients (1–6). Recently, cachexia has been defined as a metabolic syndrome associated with underlying illness and physically characterized by loss of musculature, with or without loss of fat mass (2,3). One of its prominent clinical features is weight loss (adults) and growth failure (children). Anorexia, inflammation, insulin resistance, and increased muscle protein breakdown are frequently seen in patients with cachexia and are associated with increased morbidity (3). In cancer cachexia, weight loss is observed in 30% to 80% of patients, and in 15% the condition is very severe depending on the tumor types. Stomach or pancreatic cancer patients showed weight loss at very high frequencies, whereas in patients with non-Hodgkin's lymphoma, breast cancer, acute nonlymphocytic leukemia, or sarcomas, weight loss was less prominent (5,6). Cachexia is different from starvation, age-related loss of musculature, primary depression, malabsorption, and hyperthyroidism. Despite cachexia being a critical factor in clinical therapeutic treatments, the mechanisms causing cachexia have not been fully understood and there are only a few experimental studies on the mechanisms eliciting cachexia derived from stomach cancer (7,8).

Establishment of xenograft animal models is a promising way to investigate the pathological events of this devastating disorder. Xenograft mouse models of stomach cancer, which show human-like tumor progression and cachectic behavior, help to understand the disease and develop novel therapeutic approaches. Reports of cases of orthotopic implantation of stomach cancer cells into subserous regions of the stomach of host animals have shown that the subsequent tumor growth results in peritoneal dissemination and metastases to various organs/tissues, similar to human cases (9,10).

The isoflavones used in the present study were antioxidants (11). In addition, they have anticancer effects (12) and inhibit angiogenesis (13), topoisomerase (14), and tyrosine kinase (15). In the present study, we examined whether cachectic weight loss would be observed in 15 different stomach-cancer-bearing animals. Such loss, however, was observed with low frequency only in mice implanted with the MKN-45 cell line (16). From these mice, cachexia-inducing tumor cells were removed and cultured, and then cells were implanted into naïve mice. Cells inducing cancer cachexia steadily were isolated after 10 cycles of this stepwise selection (MKN45cl85 from MKN-45). Furthermore, orthotopic implantation of MKN45cl85 cells caused ascites formation in a few of the animals. We repeated cycles of isolation of ascetic tumor cells and orthotopic reimplantation of these cells into animals to isolate highly peritoneal metastatic cells. In the same way, the 85As2 cell line was established from MKN45cl85 cells in 11 cycles of stepwise selection. The 85As2mLuc cell line is highly metastatic and cachectic. Using these cachexia-inducing mouse models, we examined the biological effects of isoflavones (genistein, daidzein, and AglyMax) on potent cancer cells in vitro and in vivo. We have reported in an in vitro and in vivo study that isoflavones inhibit the growth of several cell lines derived from human stomach cancer (17); however, these cancer cell lines are different from those newly established in this study. These 2 mouse models seem suitable for quantitative imaging studies of monitoring dynamic tumor growth as well as efficacy of anticachectic agents.

MATERIALS AND METHODS

Cell Lines and Culture

Twelve human stomach cancer cell lines (18), 10 human pancreatic cancer cell lines (19), and 5 malignant mesothelioma cell lines (20) had been established and used in our study (Table 1). Three stomach cancer cell lines (MKN-28, MKN-45, and MKN-72) were kindly provided by Dr T. Suzuki (Fukushima Medical University, Fukushima, Japan) (16). The MKN-45 cell line was established from gastric carcinoma of the poorly differentiated adenocarcinoma type from a freshly resected surgical specimen of the metastatic tumor of a 62-yr-old Japanese female patient. These cell lines were maintained in RPMI-1640 medium (GIBCO, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS; GIBCO), 100 IU/mL penicillin G sodium, and 100 mg/mL streptomycin sulfate (GIBCO). All cell lines were routinely tested for Mycoplasma using a PCR Mycoplasma Detection kit (Takara, Kyoto, Japan), and no contamination was detected.

Cytokine Assay

The levels of secreted cytokines in culture supernatants or mouse sera were determined using an ELISA kit according to the manufacturer's instructions. Human interleukin (IL)-6, IL-10, transforming growth factor (TGF)-β, and tumor necrosis factor (TNF)-α assay kits were purchased from R&D Systems (Minneapolis, MN) and human TGF-α assay kit was purchased from Oncogene (Boston, MA). In brief, cells were seeded in 6-well plates (Nunc, Roskilde, Denmark) at 1×10^5 cells per well in RPMI-1640 medium containing 10% FBS. Following overnight incubation, the culture fluid was discarded and replaced by chemically defined medium composed of DMEM/Ham's F-12 (1:1) medium supplemented with 0.05% bovine serum albumin (21). Cell number and viability were monitored using the Trypan Blue exclusion method. Triplicate wells were harvested for 2 consecutive days and the culture supernatant was transferred to 96-well plates precoated with antibodies for the cytokine assay. The supernatant-antibody reactions were processed at room temperature for 2 h. After incubation, the plates were washed 3 times with washing buffer and immersed in a solution of secondary antibodies conjugated with horseradish peroxidase (HRP) for 2 h. A chromogen (tetramethylbenzidine) was then added for yielding reaction products with a specific wavelength absorbance. H_2SO_4 (1 M) was added to stop the reaction. The plates were read in a microplate reader (Wallac 1420 ARVO MX, Perkin Elmer, Waltham, MA).

TABLE 1
Cachexia-inducing cancer cell lines assessed by body weight loss

	Cell line	Tumor forma-tion	Loss of body weight		Cell line	Tumor formation	Loss of body weight
Stomach	HSC-39	5/5	0/5	Pancreatic	Sui65	5/5	0/5
cancer	HSC-40A	5/5	0/5	cancer	Sui66	5/5	0/5
	HSC-41	5/5	0/5		Sui67	5/5	0/5
	HSC-42	5/5	0/5		Sui68	5/5	0/5
	HSC-43	5/5	0/5		Sui69	5/5	0/5
	HSC-44PE	5/5	0/5		Sui70	5/5	2/5
	HSC-45	5/5	0/5		Sui71	5/5	0/5
	HSC-57	5/5	0/5		Sui72	5/5	0/5
	HSC-58	5/5	0/5		Sui73	5/5	0/5
	HSC-59	5/5	0/5		Sui74	5/5	0/5
	HSC-60	5/5	0/5	Malignant mesothe-lioma	TCC-MESO-1	5/5	0/5
	HSC-64	5/5	0/5		TCC-MESO-3	5/5	0/5
	MKN-28	5/5	0/5		Me1Tu	5/5	0/5
	MKN-45	5/5	2/5		Me2Tu	5/5	0/5
	MKN-72	5/5	0/5		Me3Tu	5/5	0/5

Mice were implanted s.c. into left and right flank (1×10^6 cells suspended in 100 μL PBS /site). Four wk later, the mice were sacrificed and body weight loss was evaluated. Fractions indicate occurrence in the samples.

Animal Experimentation

Female BALB/c nu/nu mice were purchased from Clea Japan (Tokyo, Japan) and maintained under a specific pathogen-free condition. Six- to 8-wk-old mice were used. They were housed in filter-protected cages and reared on sterile water. The ambient light condition was controlled artificially by a 12-h light/dark cycle. All procedures involving animals and their care in this study were approved by the Committee for Ethics in Animal Experimentation of the Yasuda Women's University and the National Cancer Center Institute in accordance with institutional and Japanese Government Guidelines for Animal Experiments.

Isolation of Cachexia-Inducing Cell Lines

Following s.c. inoculation, only 10% to 20% of the MKN-45 and Sui70 tumor-bearing mice showed body weight loss. In a preliminary in vivo study of tumor growth, 1×10^6 MKN-45 cultured cells were inoculated s.c. into BALB/c nu/nu mice. After confirming tumor growth and body weight loss, the mice were sacrificed and tumor tissues were removed under sterile conditions for in vitro cultivation. The obtained specimens were washed 5 times in RPMI-1640 medium containing 500 IU/mL penicillin G sodium and 500 mg/mL streptomycin sulfate. The tumor tissues were trimmed to remove necrotic tissue debris and then minced with a scalpel. Next, 10 to 15 pieces of tissues were transferred into 60-mm culture dishes with 2 mL RPMI-1640 medium containing 20% FBS. The dishes were left undisturbed for 10 h at 37°C in a 5%CO_2/95% air atmosphere. After 10 h,

RPMI-1640 medium with 10% FBS, 100 IU/mL penicillin G sodium, and 100 mg/mL streptomycin sulfate was added to the dishes. After 7 to 14 days, cells were trypsinized (0.05% trypsin and 0.02% EDTA) to selectively remove overgrowing fibroblasts. Half the volume of the culture medium was changed on average every 4th day. After 4 to 6 wk of cultivation, the grown tumor cells (1×10^6) were reimplanted s.c. into mice. Cachexia-inducing tumor cells were removed from these mice, cultured, and then implanted into naïve mice. This process was repeated multiple times to isolate highly potent cachexia-inducing cell lines, and mice subjected to implantation with selected cells showed cachectic weight loss and body wasting. Cells inducing cancer cachexia steadily were isolated after 10 cycles of step-wise selection. The newly established cell line MKN45cl85 was used in the present study.

Analysis of Tumorigenicity and Tumor Growth

The tumorigenicity and cachexia-inducing ability of MKN-45 and MKN45cl85 cell lines were tested by s.c. injection into mice (1×10^6 cells suspended in 100 μL PBS). The tumor was measured in 2 dimensions with calipers, and the volume of each tumor was calculated according to the equation $(L \times W^2)/2$ ($L =$ length, $W =$ width). At intended schedules of survival, or when moribund, the mice were sacrificed and various organs were macroscopically examined for metastasis and then processed for histological examination, as described previously (19).

Establishment of Cachexia-inducing Peritoneal Metastatic Cell Lines

The MKN45cl85 cell line (1×10^6/50 μL) was implanted orthotopically under the serosal membrane in the greater curvature of the antrum in BALB/c nu/nu mice, according to a previously described in vivo stepwise selection method (10,22). After 8 wk, the mice were killed and autopsied. The stomach, regional lymph nodes, liver, and other organs were resected to evaluate the metastatic potential of this cell line and processed for routine histopathological examination. Orthotopic implantation of MKN45cl85 cells caused ascites formation in a few animals. We repeated cycles of isolation of ascetic tumor cells and orthotopic reimplantation of these cells into animals to isolate highly metastatic cells with a strong capability of inducing peritoneal dissemination. In the same way, the 85As2 cell line was established in 11 cycles of stepwise selection.

In Vivo Photon Counting Analysis

First, the luciferase gene was inserted into the MSCVIRES-GFP vector. To generate an RD114-pseudotyped retrovirus, the Lipofectamine-Plus reagent (Invitrogen, Carlsbad, CA) was used to transfect 293T cells with the expression vectors for luciferase, pEQ-PAM3(-E), and pRDF (obtained from the St. Jude Vector Development and Production Shared Resource, Memphis, TN). Conditioned medium containing the retroviruses was harvested from transfected 293 cell cultures and stored at -80°C until use. 85As2 cells were transformed in virus-conditioned medium with 4 mg/mL polybrene (Sigma-Aldrich, St Louis, MO) in retronectin-precoated wells (Takara, Kyoto, Japan). Transformed cells showing high GFP expression levels were separated using a FACS Aria cell sorter (Becton Dickinson, Franklin Lakes, NJ). The competent 85As2 cells carrying the luciferase gene was named 85As2mLuc.

Second, orthotopic implantation of 1×10^6 85As2mLuc cells was conducted in 8-wk-old female BALB/c-nu/nu mice (day 0) as described previously (10). To assess the tumorigenicity of 85As2mLuc cells, bioluminescence signals from 85As2mLuc cells implanted into mice were monitored after i.p. injection of D-luciferin (150 mg/kg) using the IVIS system Lumina series (Caliper Life Sciences, Hopkinton, MA) as described previously (20,22). During the entire course of monitoring, the animals were kept under isoflurane anesthesia. The tumor volume was identified as the regions of interest (ROI) of bioluminescence and the total photon counts were quantified using the Living Image acquisition and Analysis Software (Caliper Life Sciences). The tumor volume (or ROI) was measured every 7th day and the proliferative growth was determined on the basis of bioluminescence signals by subtracting the value on postimplantation day 1. The tumor volume in each group was expressed as the average values of the total photon counts (22). At the end of the in vivo study, various organs were dissected, examined macroscopically for metastatic foci, and processed for histopathological examination.

Effect of Isoflavones on Tumor Cell Growth In Vitro and In Vivo

Daidzein and genistein were purchased from Sigma-Aldrich and Nagara Science (Gifu, Japan). Isoflavone aglycone AglyMax (Nichimo Co, Tokyo, Japan) was prepared from soybeans fermented with Koji fungus (*Aspergillus awamori*) and consisted of daidzein, genistein, and glycitein aglycone at a ratio of 7:1:2 (23).

To examine the effects of these isoflavones on in vitro proliferation of cancer cells, each flavonoid compound was applied onto 1-day cultures of MKN45cl85 and 85As2mLuc cells seeded initially in 24-well plates at 1×10^4 cells/mL per well. Isoflavones were first dissolved in DMSO and then used by diluting in culture medium (final concentrations: 100 μM daidzein, 200 μM genistein, and 200 μM AglyMax). On days 2 to 5, cultured cells were trypsinized, collected, and counted to assess the bioactive effects on cell proliferation. The growth rate was also determined by using a crystal violet dye elution (CVE) assay, as described previously (17). An equal volume of DMSO was added to control cultures and no measurable effect was detected on cell growth.

In experiments using either the MKN45cl85 s.c. implantation model or the 85As2mLuc orthotopic implantation model, AglyMax, daidzein, and genistein (400 μg/0.1 mL per head per day) were injected i.p. every other day starting 2 days before implantation surgery (12 times in total). Control animals were given the same vehicle (DMSO). Tumor growth (s.c.) was measured weekly with calipers (as tumor diameter). The 85As2mLuc tumor growth was monitored by in vivo photon counting analysis 1 day after orthotopic implantation. Food and water were available ad libitum. The mice were observed daily for clinical signs and mortality. Body weight and food consumption were measured weekly. Three or 4 wk later, the mice were sacrificed and tumors grown in the hosts were removed and weighed.

Statistical Analysis

All numerical data are expressed as means ± SE. Data of cytokine levels in culture supernatants or mouse sera, tumor size, and body weight were analyzed using Student's t-test. The survival rate of mice in in vivo experiments was analyzed by Cox-Mantel test. P values less than 0.05 were considered statistically significant.

RESULTS

Isolation of Cachexia-inducing Stomach Cancer Cell Lines

Table 1 shows cancer cachexia-inducing cell lines in terms of body weight loss in mice. Tumor growth was observed in all mice bearing a xenograft, but cachectic weight loss was observed in mice implanted with MKN-45 stomach cancer cells, as well as Sui70 pancreatic cancer cells (19). A highly competent cachexia-inducing tumor cell line was only isolated from the MKN-45 human stomach cancer cell line according to the

protocol described in the **Materials and Methods** section and labeled MKN45cl85.

Subcutaneous implantation of MKN45cl85 cells induced rapid tumor growth in mice as early as 3 wk after surgery (Fig. 1A). The weight loss caused by cancer cachexia set out as the tumor growth plateaued. The mice appeared to get into a cachectic condition, accompanied by decreased activity, xeroderma, and anorexia in 90% to 100% of the mice within 3 to 5 wk postimplantation (Table 2). The initial sign of cachexia depends on the number of inoculated cells. Tumor tissues grown as large as 10 mm in diameter resulted in critical conditions of cancer cachexia in mice, and surgical removal of the tumors resulted in the restoration of healthy conditions (Fig. 1B and 1C). Moreover, the xenograft of MKN45cl85 cells in the mice reduced adipose tissue and musculature volumes (Fig. 1C, lower panel).

Isolation of Cachexia-inducing Peritoneal Metastatic Cell Lines

The MKN45cl85 cancer cell line described above had no metastatic competence to peritoneal dissemination. In xenograft experiments of MKN45cl85 cells, ascitic tumors developed in 20% to 30% of host mice. Accordingly, another cancer cell line was newly isolated from these ascitic tumors of mice by repeated isolation of competent metastatic cells in vitro and subsequent xenografting in mouse. The new metastatic cell line named 85As2, which is derived from MKN45cl85 cancer cells, possessed a high capability for inducing peritoneal dissemination (Table 2). For further study, the luciferase gene was introduced into 85As2 tumor cells and the resultant cells were named 85As2mLuc. 85As2mLuc cells were able to cause cancer cachexia in 100% of mice after 3 to 5 wk, by either orthotopic or subcutaneous implantation (Fig. 1D). The musculature and adipose tissue weights in cachectic mice at 4 wk after subcutaneous implantation were remarkably and significantly reduced compared with those of the nontumor-bearing group [gastrocnemius muscle (g): nontumor-bearing, 0.1252 ± 0.0074, $n = 5$, and tumor-bearing, 0.0757 ± 0.0049, $n = 5$, $P < 0.001$; epididymal fat (g): nontumor-bearing, 0.1056 ± 0.0181, $n = 5$ and tumor-bearing, 0.0000 ± 0.0000, $n = 5$).

Characterization of 2 Cachexia-inducing Cancer Cell Lines

The MKN45cl85 cell line, as well as the parental MKN-45 cell line, had round or spindle-shaped-like cell morphology, tended to aggregate loosely, and showed a characteristic pattern of attaching to the bottom of the flask. On the other hand, 85As2mLuc cells were round and nonadherent (Fig. 2, upper column). A clear difference was observed with regard to adhesion to the bottom of the dishes.

The supernatants collected from MKN45cl85, 85As2mLuc, and MKN-45 cell line cultures were processed for examining cachectic cytokines produced by the cells (Table 2). IL-6, IL-10, TGF-α, TGF-β, and TNF-α were not detected in all cell lines. In addition, these cytokines were not detected in the sera of cachectic mice that received s.c. implantation of MKN45cl85 and 85As2mLuc cells.

The number of postimplantation survival days of mice was relatively smaller in 85As2mLuc cells than in MKN45cl85 and MKN-45 cells (Table 2). When the 3 cancer cell lines were implanted s.c., mice developed tumors in situ but exhibited no metastasis to other organs. On the other hand, orthotopic implantation of the 3 cell lines caused metastases to various organs. Of the 3 cell lines, only 85As2mLuc cancer cells caused peritoneal dissemination of the cells to the mesentery and omentum in mice. Metastases of 85As2mLuc cancer cells to neighboring lymph nodes and liver were also frequently observed (Table 2). Mice implanted with 85As2mLuc cells appeared to be more severely affected than those receiving MKN45cl85 and MKN-45 cells.

Effects of Isoflavones on Proliferation of Cachexia-inducing Cancer Cells In Vitro

Effects of the 3 types of isoflavones on cancer cell proliferation were examined using MKN45cl85 and 85As2mLuc cell lines in vitro (Fig. 2, lower column). After application of the flavonoid compounds, cell proliferation was monitored on a daily basis, using the CVE assay. The inhibitory effects of genistein and AglyMax on proliferation of both cancer cell lines were observed at 1 μM at 72 h after application of the compounds. The inhibitory effects increased as the concentrations of the compounds increased (up to 200 μM). Daidzein was relatively less effective because it could inhibit proliferation of both cancer cell lines at a concentration as high as 100 μM (Fig. 2, lower column). This inhibitory effect of daidzein appeared from 24 h through 96 h after application of the compound, with a gradual increase in the inhibition as time proceeded. Thus, the 3 isoflavones exerted inhibitory effects on proliferation of the 2 cachexia-inducing cancer cell lines.

Suppressive Effects of Isoflavones on Tumor Growth and Cachexia In Vivo

The antineoplastic and anticachectic activities of the isoflavones were investigated using 2 xenograft implantation models leading to cancer cachexia (Table 3 and Fig. 3). First, effects of 3 types of isoflavones on tumor growth and cachexia were examined in mice that underwent s.c. implantation of MKN45cl85 cells (Fig. 3A and 3B). Of the 3 flavonoid compounds, AglyMax significantly inhibited proliferative growth of the xenografted cells (Fig. 3A). Daidzein and genistein suppressed the proliferation of cancer cells to a lesser extent in mice. The extent of the inhibitory effect of isoflavones against tumor growth was graded as AglyMax > daidzein > genistein (Fig. 3A). We also examined changes in body weight in these isoflavone-treated mice. The anticachectic activities of the 3 isoflavones in terms of the body weight loss in these s.c. implantation mouse models are shown in Fig. 3B. AglyMax- and daidzein-treated mice did not show cachexia-associated body
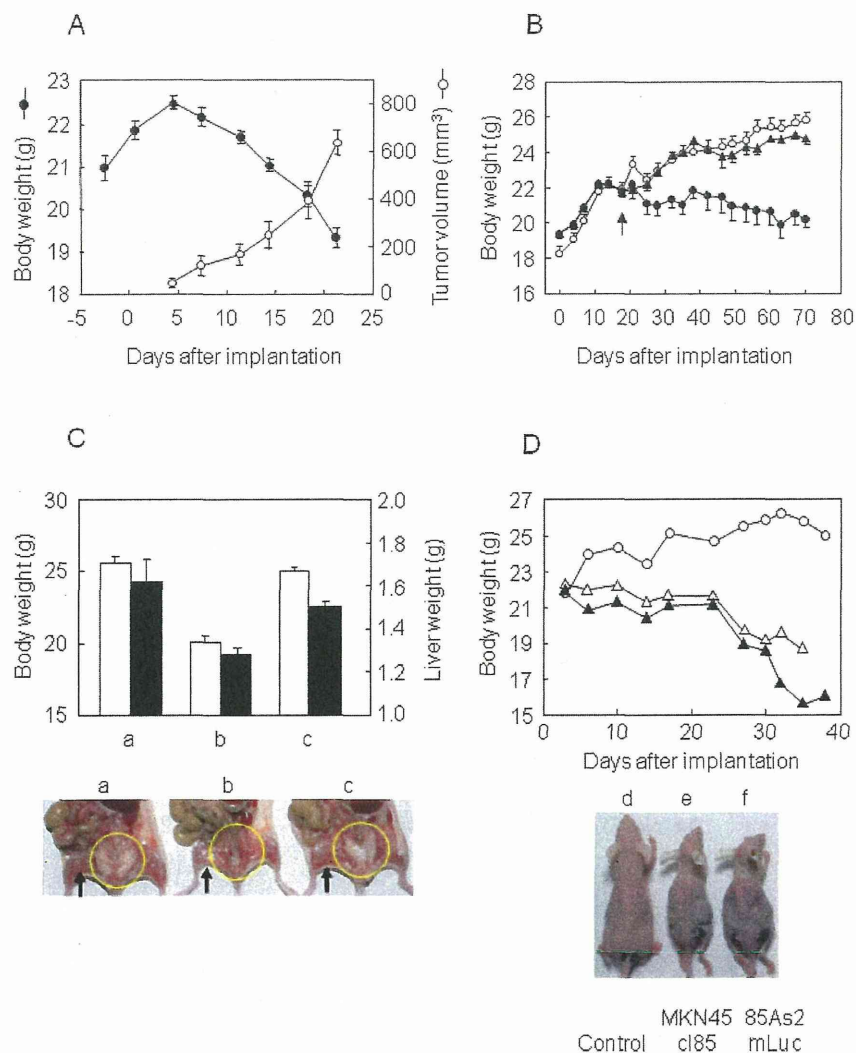
FIG. 1. Body weight and tumor growth of mice bearing MKN45cl85 and 85As2mLuc cancer cell lines. **A:** Growth curves of body weight of mice and tumor volume. MKN45cl85 cells were s.c. implanted into left and right flanks (1×10^6 cells/each site) on Day 0. Body weight (•) and tumor volume (o) were measured periodically. $n = 5$. **B:** Growth curves of body weight of mice. MKN45cl85 cells were implanted s.c. into the right flank (1×10^6 cells/site) on Day 0. One group of mice carried the tumor during the experiment (•). In another group (▲), tumors that grew as large as 10 mm in diameter were removed on Day 18 (arrow). The control group consisted of age- and sex-matched mice (o). $n = 5$. **C:** Body weight (open bar) and liver weight (solid bar) in histograms and macroscopic views of autopsies of the lower halves of mouse bodies at 3 wk after s.c. implantation of MKN45cl85 cells. In both upper and lower panels, (a) age- and sex-matched control, (b) tumor bearing, and (c) tumor-removed mice. Apparent changes in the volume of the parametrical fat mass (yellow circle) and hind limb musculature (arrow) were recognized. $n = 5$. **D:** Body weight curves and dorsal views of mice that underwent orthotopic implantation of MKN45cl85 (△, e) and 85As2mLuc (▲, f) cancer cells (1×10^6 cells) into subserous regions of the stomach on day 0. Control mice were age- and sex-matched (o, d) (Color figure available online).

weight loss, but cachectic weight loss was observed in genistein-treated mice.

Next, we examined tumor growth and cachexia-suppressive activities of isoflavones in mice, in which 85As2mLuc cells were implanted orthotopically. This mouse model supported the results obtained in the experiment of s.c. implantation of MKN45cl85 cells (Table 3 and Fig. 3). The extent of the antitumor effect of the isoflavones was graded as AglyMax >

daidzein > genistein (Fig. 3C and 3E). Furthermore, mitosis of these isoflavone-treated 85As2mLuc tumors was observed histopathologically. The mitotic index was dependent on the antitumor potential of each isoflavone (Table 3).

With regard to the characteristics of the mouse model in which 85As2mLuc cells were implanted orthotopically followed by treatment with AglyMax and daidzein, cancer cachexia was not observed and neither was body weight loss, decreased

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TABLE 2
Biological properties of newly isolated cachexia-inducing sublines and parent MKN-45 cell line

Cell line	Production of cytokine[†]					Xenotransplantation in nude mice					
									Metastasis[§]		
	IL-6 (ng)	IL-10 (ng)	TNF-α (ng)	TGF-α (pg)	TGF-β1 (ng)	Cachexia[‡] induction	Tumor[‡] formation	Survival[‡] time (days)	Peritoneal dissemination	Ascites formation	Liver/ lymph node
MKN-45	ud	ud	ud	ud	ud	3/10	10/10	38.8 (22–55)	2/10	0/10	0/10
MKN45cl85	ud	ud	ud	ud	ud	9/9	9/9	40.5 (23–53)	6/10	0/10	0/10
85As2mLuc	ud	ud	ud	ud	ud	10/10	10/10	23.3 (15–37)	10/10	7/10	8/10

ud = undetectable, below the minimum detection limit of the assay kit.

[†]Supernatant was collected from 48 h incubation cultures. Data of interleukin (IL)-6, IL-10, tumor necrosis factor (TNF)-α, and TGF-β1 are expressed as ng/10^6 cells; data of TGF-α are expressed as pg/10^6 cells.

[‡]Mice were implanted s.c. into left and right flank (1 × 10^6 cells/site).

[§]Mice were implanted orthotopically into subserous regions of the stomach (1 × 10^6cells). Data of the occurrence are shown by fractions; numerators of each fraction indicate positive numbers in the samples (denominators).

activity, skin dryness, and anorexia. The mean body weights in the AglyMax-treated, daidzein-treated, and control group were 20.74 ± 0.47, 19.50 ± 0.75, and 17.24 ± 0.19 g, respectively, on Day 26 (Table 3 and Fig. 3D, $P = 0.05$). Histological examinations of coelomic viscera (liver, lung, heart, kidney, adrenal gland, and ovary) of AglyMax- and daidzein-treated mice showed that they were normal. In contrast, body weight loss was seen in both vehicle- and genistein-treated mice.
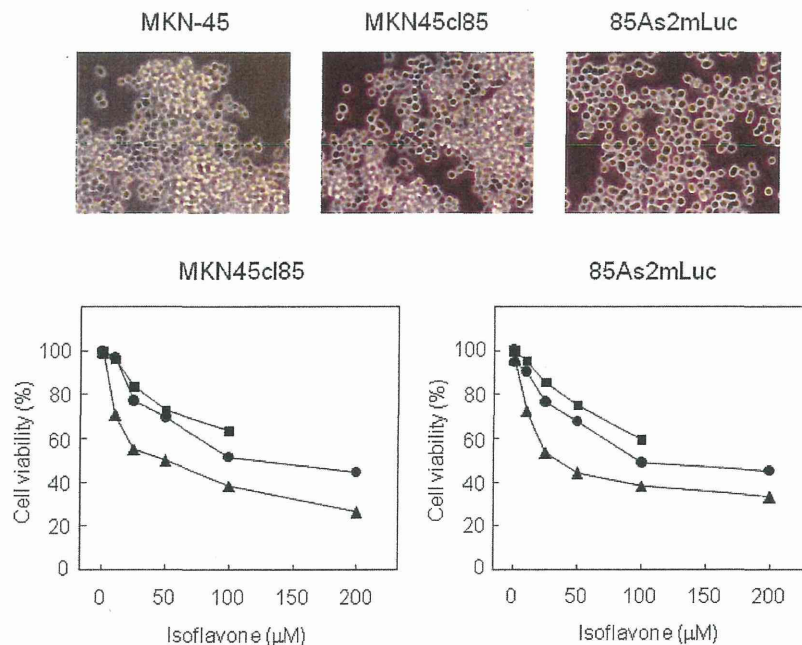

FIG. 2. Phase-contrast photomicrographs and dose-dependent viability curves of cachexia-inducing cancer cell lines under inhibitory influence of 3 types of isoflavones. The inhibitory effects of isoflavones on the viability of cancer cells were dose-dependent. Original magnification, ×200. MKN45cl85 and 85As2mLuc cells were initially seeded at 1 × 10^4 cells/mL per well, incubated overnight, and then exposed to different types of isoflavones, genistein (▲), daidzein (■) and AglyMax (●). After 74 h, cell viability was determined by using a crystal violet dye elution assay. A higher concentration (200 μM) of daidzein was not available because of its poor solubility. (Color figure available online).

TABLE 3

Effects of various isoflavones on tumor growth and body weight in orthotopic implantation: 85As2mLuc model

Isoflavones	Tumor growth[†]		
	Volume[‡] photon counts ($\times 10^8$)	Mitotic[§] index (%)	Body weight (g)
Control	34	4.61	17.24 ± 0.19
Genistein	17	3.56	18.00 ± 0.31
Daidzein	8	2.59	19.50 ± 0.75
AglyMax	5.9	0.96	20.74 ± 0.47

[†]Mice were sacrificed on Day 26 after orthotopic implantation into subserous regions of the stomach (2×10^6 cells).

[‡]Tumor volume was measured by in vivo photon counting analysis.

[§]Mitotic index of the stomach tumor was calculated using histological section (No. of mitotic cells/2000 tumor cells).

Student's t-test: daidzein group vs. control group, $P < 0.05$; Agly-Max group vs. control group, $P < 0.01$. $n = 5$.

Effects of Isoflavones on Survival and Metastasis in the Model of Orthotopic Implantation of 85As2mLuc Cells

Effects of isoflavones on survival and metastasis were examined in the 85As2mLuc model with metastatic stomach cancer, in which the tumor take rate was 100%, followed by vigorous tumor growth and subsequent death until 60 days after tumor implantation without treatment of isoflavones. The highly aggressive nature of the tumor is summarized in Table 2. Administration of isoflavones to 85As2mLuc tumor-bearing mice resulted in prolonged survival (Table 4). Metastasis of 85As2mLuc tumors to remote organs/tissues was not inhibited in mice. The volume of ascites from mice on sacrifice was similar between isoflavone- and DMSO-treated control groups. However, treatments with different isoflavones improved the median survival rate of mice in comparison with those treated with vehicle (Table 4). The effects on the survival rate of mice with regard to efficacy of isoflavones were graded as AglyMax > daidzein > genistein (Fig. 3F).

DISCUSSION

Stomach cancer ranks second in incidence and mortality worldwide, despite its overall decline (24). Asian countries with a particularly high incidence of this cancer include Japan, Korea, and China. Stomach cancer patients have a high incidence of cachexia (incidence of weight loss, about 83%) (25). Obtaining greater knowledge about the pathophysiology of cachexia for stomach cancer is urgently needed to establish methods for early diagnosis and effective treatment, including effective anticachectic therapy, of this debilitating disease. Toward this end, extensive in vivo or in vitro studies using suitable experimental models are indispensable. Animal models would be useful for analyzing cancer-associated events inside the body and for evaluating the pharmacodynamic effects of candidate anticachectic agents, if they mimicked human cases of cachexia. The orthotopic implantation mouse models of stomach cancer-derived tumors established in the present study demonstrated the development of cancer cachexia and the metastasis of these tumors to various sites at high frequency and the development of ascites within a relatively short period. These mouse models also showed a favorable response to anticancer agents, isoflavones, which may pave the way for designing manipulations to clarify the pathogenesis of cancer cachexia.

In vivo studies of cachexia of stomach cancer may be expected to play a pivotal role in understanding the nature and behavior of the tumor cells inside the body, in particular with regard to interaction with the host. We isolated a cachexia-inducing (as body weight loss) subline with nonmetastatic potential, MKN45cl85, from the stomach cancer cell line MKN-45. Moreover, we were able to isolate another cell line, 85As2mLuc, which exhibited very aggressive growth in mice, accompanied by the induction of cancer cachexia. 85As2mLuc tumors are usually more invasive and highly metastatic and associated with poor prognosis. Xenograft mouse models have attracted attention as useful cachexia models because they reflect the malignant stages of stomach cancer in the clinical setting.

Stomach cancer patients have high incidence of cachexia; however, there are few experimental cachexia models using human stomach cancer. In the present study, we screened 15 different human stomach cancer cell lines and then established 2 cachexia-inducing cancer cell lines (MKN45cl85 and 85As2mLuc) from MKN-45. It is speculated that the differences in the potency to cause cancer cachexia between these 2 cell lines and other stomach cancer cell lines may be associated with the production of the cachectic factors such as cytokines by cancer cells or host cells. In humans and rodent models, there is increasing evidence that cytokines, including TNF-α, IL-1, IL-6, IL8, IL-10, TGF-α, TGF-β, and vascular endothelial growth factor (VEGF) may be involved in the cause or development of cancer cachexia (26–32). Some investigators have found that higher IL-6 levels in patients with nonsmall cell lung, pancreatic, and prostatic cancers were associated with weight loss (33–35). In Phase I and II trials, treatment with a humanized anti-IL-6 antibody ameliorated nonsmall cell lung cancer-related cachexia (36). In addition, a human–mouse chimeric monoclonal antibody, which blocks human IL-6 but not mouse IL-6, ameliorated body weight loss in cancer cachexia mouse models inoculated with human melanoma cells or human prostate tumor cells (37). Thus, these studies have shown that IL-6 may be the cause or the potential therapeutic targeting of cancer cachexia. In the present study, secretion of IL-6 was not detected in the culture supernatants and sera of MKN45cl85 and 85As2mLuc tumor-bearing cachectic mice. On the other hand, other data have raised doubts as to whether IL-6 was an independent cachectic factor (38). Therefore, these results suggest that other factors but not IL-6 may be involved in cachexia development of the present model (39,40). Further study is necessary to investigate which
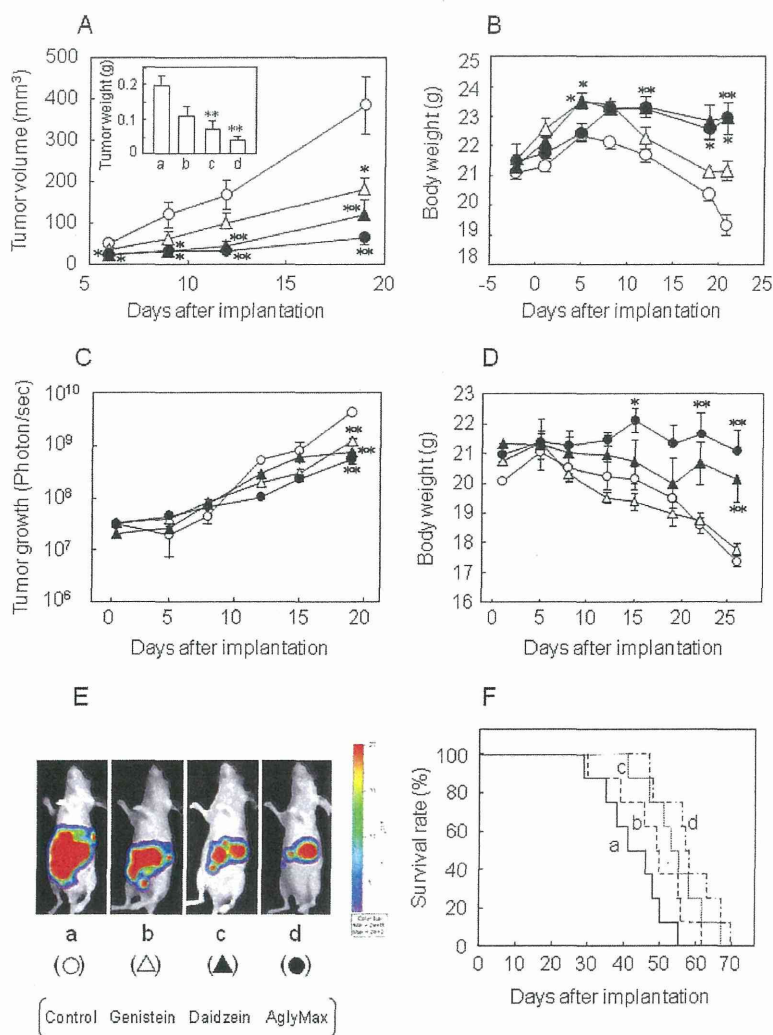
FIG. 3.   Inhibition of tumor growth and body weight loss by isoflavones in mice bearing cachexia-inducing MKN45cl85 and 85As2mLuc cells. Growth curves of tumors (A, C, and E), body weight (B and D), and days of survival (F) in mice in which MKN45cl85 cells were implanted (s.c.) into right and left flanks (1×10^6 cells/each site) and in which 85As2mLuc cells were implanted orthotopically. $n = 6$. Cancer cells were implanted on Day 0. A: Growth of tumor volume (tumor weight, inset) of MKN45cl85 cells in mice was inhibited and (B) their body weight increased by administering isoflavones (400 μg/mouse per day) i.p. every other day, starting from 2 days before implantation. Twelve shots during the experiment (4.8 mg/head). Genistein ($\triangle$, b), daidzein ($\blacktriangle$, c), and AglyMax ($\bullet$, d). Control mice received equal amounts of vehicle ($\circ$, a). C: Growth curves of tumor size and (D) body weight in mice that received orthotopic implantation of 85As2mLuc cells (2×10^6 cells) into subserous regions of the stomach on Day 0. Growth of 85As2mLuc cell tumors was measured by using the IVIS system. Mice: control ($\circ$, a), genistein ($\triangle$, b), daidzein ($\blacktriangle$, c), and AglyMax ($\bullet$, d). *$P < 0.05$, **$P < 0.01$ compared with the values for the control on the corresponding days. E: Photographs showing in vivo quantitative photon counting analysis utilizing bioluminescence signals on day 26 after orthotopic implantation of 85As2mLuc cells. Isoflavones were administered i.p. every other day, starting from 2 days before implantation. The size of tumors in mice was indicated by color (red is intense and blue is the opposite). Mice: control ($\circ$, a) genistein ($\triangle$, b), daidzein ($\blacktriangle$, c), and AglyMax ($\bullet$, d). F: A chart showing days of survival in mice after orthotopic implantation of 85As2mLuc cancer cells (1×10^6 cells) in subserous regions of the stomach. Surgery was carried out on Day 0. Isoflavones were administered i.p. every other day, starting from 2 days before implantation. The efficacy of different types of isoflavones on survival in mice was graded in intensity. (a) vehicle, (b) genistein, (c) daidzein, and (d) AglyMax. The groups consisted of 8 mice each. Cox-Mantel test: daidzein group vs. control group, $P = 0.06$; AglyMax group vs. control group, $P = 0.01$. (Color figure available online).

cachectic factors contributed to the different potency to cause cachexia.

Epidemiologic, in vitro, and in vivo studies have provided evidence for the hypothesis that certain soy phytochem-icals, especially soy isoflavones, have antitumorigenic properties (12–15,17,41,42). In the present study, we have assessed the anticachexia effects of isoflavones using 2 stomach cancer models. We demonstrated that administration of isoflavones

TABLE 4
Effect of various isoflavones on survival and metastasis in an orthotopic implantation: 85As2mLuc model

Isoflavones	Survival[†] time (days)	Metastasis[‡]			
		Ascites[§] formation	Peritoneal dissemination	Lymph node (regional)	Liver surface
Control	42.8 (29–55)	6/8	8/8	6/8	6/8
Genistein	48.4 (30–62)	5/8	8/8	5/8	5/8
Daidzein	54.3 (41–67)	6/8	8/8	5/8	5/8
AglyMax	58.3 (47–70)	5/8	8/8	5/8	5/8

[†]Mice were implanted orthotopically into the subserous regions of stomach cancer cells (1×10^6 cells). Mice were sacrificed on day 70 after implantation or when moribund.

[‡]Data of the metastasis are shown by fraction; numerators of each fraction indicate positive numbers in the samples (denominators).

[§]Ascites formation and body weight were assessed at the time of death (body weight includes ascites).

mitigated cachexia and prolonged the survival of the host by mechanisms mainly dependent of their antiproliferative action. However, the mechanism causing the shorter survival associated with cachexia has not been clarified yet. Suppression of cancer cachexia may be due to inhibition of tumor proliferation by isoflavones. We have previously found that 11 cultured human stomach cancer cell lines undergo morphological changes and internucleosomal DNA fragmentation characteristic of apoptosis after exposure to isoflavone derivatives (biochanin A, daidzein, and genistein) in vitro and in vivo (17). We and others have also shown that these isoflavones induce apoptosis in a specific cell cycle phase (43,44). Biochanin A and daidzein induce G_1 arrest, whereas genistein blocks at G_2-M in cancer cell lines (43). Accordingly, our observations indicate that 3 isoflavones (AglyMax, daidzein, and genistein) mitigate cachexia and inhibit tumor proliferation of stomach cancer cell lines in vivo through activation of a signal transduction pathway for apoptosis. Moreover, quercetin, a flavonoid compound abundantly present in fruits, vegetables, wine, and tea has several activities such as antiproliferative and anticancer activities. Camargo et al. (45), showed inhibition of tumor growth, prolonged survival, decrease in matrix metalloproteinase-2 and VEGF levels, and reduction of cachexia in walker 256 tumor-bearing rats treated with quercetin. Their results strongly support the anticancer function, including cachexia, of this flavonoid compound. We hypothesize that increased intake of soy bioactive components may provide an effective nutritional regimen for the prevention of stomach cancer progression because soy bioactive components can exert their effects via both blood circulation and, in particular, direct contact with the mucosa of the stomach.

On the other hand, during the past decade, increasing evidence has shown that dysregulation of inflammatory pathways contributes to the development of cachexia (46). The nuclear factor (NF)-κB is a major mediator of inflammatory pathways. Therefore, antiinflammatory agents that can modulate NF-κB activation and inflammatory pathways may have potential in improving cancer-related symptoms such as cachexia (47, 48).

More recently, studies using in vitro and in vivo models have demonstrated that isoflavones inhibit NF-κB activation and modulate inflammatory pathways (49). Further investigations are needed to elucidate the mechanisms responsible for cancer cachexia.

In conclusion, we isolated 2 cachexia-inducing sublines (MKN45cl85 and highly metastatic 85As2mLuc) from the human stomach cancer cell line MKN-45. These cell lines may provide a good tool to study the development of cancer cachexia and malignant progression of human stomach cancer. To our knowledge, the MKN45cl85 and metastatic 85As2mLuc cell lines are the first stomach cancer cell lines reported to induce cachexia at high frequency in mice. These cachectic animal models are worthy of further study to explore the treatment of stomach cancer-induced cachexia. Our results demonstrated that isoflavones could exert therapeutic efficacy on amelioration of cancer cachexia in the host. Nevertheless, the results of this study also indicate that a longer period of treatment is required to maintain these effects.

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REFERENCES

1. Loberg RD, Bradley DA, Tomlins SA, Chinnaiyan AM, and Pient KJ: The lethal phenotype of cancer: the molecular basis of death due to malignancy. *CA Cancer J Clin* **57**, 225–241, 2007.

2. Fearon K, Strasser F, Anker SD, Bosaeus I, Bruera E, et al.: Definition and classification of cancer cachexia: an international consensus. *Lancet Oncol* **12**, 489–495, 2011.

3. Evans WJ, Morley JE, Argiles J, Bales C, Baracos V, et al.: Cachexia: A new definition. *Clin Nutr* **27**, 793–799, 2008.

4. Muscaritoli M, Bossola M, Aversa Z, Bellantone R, and Fanelli FR: Prevention and treatment of cancer cachexia: new insights into an old problem. *Eur J Cancer* **42**, 31–41, 2006.

5. Dewys WD: Weight loss and nutritional abnormalities in cancer patients: incidence, severity and significance. In: *Clinics in Oncology*, Calman KC, Fearon KCH, eds. London: Saunders, 1986, 251–261.

6. Laviano A, Meguid MM, Inui A, Muscaritoli M, and Rossi-Fanelli F: Therapy insight: cancer anorexia-cachexia syndrome-when all you can eat is yourself. *Nat Clin Prac Oncol* **2**, 158–165, 2005.

7. Mori T, Fujiwara Y, Yano M, Tamura S, Yasuda T, et al.: Prevention of peritoneal metastasis of human gastric cancer cells in nude mice by S-1, a novel oral derivative of 5-fluorouracil. *Oncology* **64**, 176–182, 2003.

8. Tanaka F, Tominaga K, Shiota M, Ochi M, Kuwamura H, et al.: Interleukin-10 gene transfer to peritoneal mesothelial cells suppresses peritoneal dissemination of gastric cancer cells due to a persistently high concentration in the peritoneal cavity. *Cancer Gene Ther* **15**, 51–59, 2008.

9. Fidler IJ, Naito S, and Pathak S: Orthotopic implantation is essential for the selection, growth and metastasis of human renal cell cancer in nude mice. *Cancer Metastasis Rev* **9**, 149–165, 1990.

10. Yanagihara K, Takigahira M, Tanaka H, Komatsu T, Fukumoto H, et al.: Development and biological analysis of peritoneal metastasis mouse models for human scirrhous stomach cancer. *Cancer Sci* **96**, 323–332, 2005.

11. Wei H, Cai Q, and Rahn RO: Inhibition of UV light- and Fenton reaction-induced oxidative DNA damage by the soybean isoflavone genistein. *Carcinogenesis* **17**, 73–77, 1996.

12. Booth C, Hargreaves DF, Hadfield JA, McGown AT, and Potten CS: Isoflavones inhibit intestinal epithelial cell proliferation and induce apoptosis in vitro. *Br J Cancer* **80**, 1550–1557, 1999.

13. Fotsis T, Pepper M, Adlercreutz H, Fleischmann G, Hase T, et al.: Genistein, a dietary-derived inhibitor of in vitro angiogenesis. *Proc Natl Acad Sci* **9**, 2690–2964, 1993.

14. Okura A, Arakawa H, Oka H, Yoshinari T, and Monden Y: Effect of genistein on topoisomerase activity and on the growth of [Val 12]Ha-ras-transformed NIH 3T3 cells. *Biochem Biophys Res Commun* **30**, 183–189, 1988.

15. Akiyama T, Ishida J, Nakagawas S, Ogawara H, Watanbe S: Genistein, a specific inhibitor of tyrosine-specific protein kinase. *J Biol Chem* **25**, 5592–5595, 1987.

16. Motoyama T, Hojyo H, and Watanabe E: Comparison of seven cell lines derived from human gastric carcinomas. *Acta Path Jap* **36**, 65–83, 1986.

17. Yanagihara K., Ito A, Toge T, and Numoto M: Antiproliferative effects of isoflavones on human cancer cell lines established from the gastrointestinal tract. *Cancer Res* **53**, 5815–5821, 1993.

18. Yanagihara K, Tanaka H, Takigahira M, Ino Y, Yamaguchi Y, et al.: Establishment of two cell lines from human gastric scirrhous carcinoma that possess the potential to metastasize spontaneously in nude mice. *Cancer Sci* **95**, 575–582, 2004.

19. Yanagihara K, Takigahira M, Tanaka H, Arao T, Aoyagi Y, et al.: Establishment and molecular profiling of a novel human pancreatic cancer panel for 5-FU. *Cancer Sci* **99**, 1859–1864, 2008.

20. Yanagihara K, Tsumuraya M, Takigahira M, Mihara K, Kubo T, et al.: An orthotopic implantation mouse model of human malignant pleural mesothelioma for in vivo photon counting analysis and evaluation of the effect of S-1 therapy. *Int J Cancer* **126**, 2835–2846, 2010.

21. Yanagihara K and Tsumuraya M: Transforming growth factor β_1 induces apoptotic cell death in cultured human gastric carcinoma cells. *Cancer Res* **52**, 4042–4045, 1992.

22. Yanagihara K, Takigahira M, Takeshita F, Komatsu T, Nishio K, et al.: A photon counting technique for quantitatively evaluating progression of peritoneal tumor dissemination. *Cancer Res* **66**, 7532–7539, 2006.

23. Xu JW, Yasui N, Ikeda K, Pan WJ, Watanabe J, et al.: Isoflavones regulate secretion of leukemia inhibitory factor and transforming growth factor-β and expression of glycodelin in human endometrial epithelial cells. *J Endocrinology* **196**, 425–433, 2008.

24. Parkin DM, Bray F, Ferlay J, and Pisani P: Estimating the world cancer burden: Globocan 2000. *Int J Cancer* **94**, 153–156, 2001.

25. Bruera E: ABC of palliative care. Anorexia, cachexia and nutrition. *Br Med J* **315**, 1219–1222, 1997.

26. Tisdale MJ: Mechanisms of cancer cachexia. *Physio Rev* **89**, 381–410, 2009.

27. Krzystek-Korpacka M, Matusiewicz M, Diakowska D, Grabowski K, Blachut K, et al.: Impact of weight loss on circulating IL-1, IL-6, IL-8, TNF-α, VEGF-A, VEGF-C and midkine in gastroesophageal cancer patients. *Clin Biochem* **40**, 1353–1360, 2007.

28. Chang JW, Yeh KY, Shen YC, Hsieh JJ, Chuang CK, et al.: Production of multiple cytokines and induction of cachexia in athymic nude mice by a new anaplastic thyroid carcinoma cell line. *J Endocrinology* **179**, 387–394, 2003.

29. Kumar S, Kishimoto H, Chua HL, Badve S, Miller KD, et al.: Interleukin-1α promotes tumor growth and cachexia in MCF-7 xenograft model of breast cancer. *Am J Pathol* **163**, 2531–2541, 2003.

30. Kamoshida S, Watanabe K, Suzuki M, Mizutani Y, Sakamoto K, et al.: Expression of cancer cachexia-related factors in human cancer xenografts: an immunohistochemical analysis. *Biomed Res* **27**, 275–281, 2006.

31. Fujimoto-Ouchi K, Tamura S, Mori K, Tanaka Y, and Ishitsuka H: Establishment and characterization of cachexia-inducing and non-inducing clones of murine colon 26 carcinoma. *Int J Cancer* **61**, 522–528, 1995.

32. Kemik O, Kemik AS, Begenik H, Erdur FM, Emre H, et al.: The relationship among acute-phase response proteins, cytokines, and hormones in various gastrointestinal cancer types patients with cachectic. *Hum Exp Toxicol* **31**, 117–125, 2012.

33. Scott HR, McMillan DC, Crilly A, MaArdle CS, and Milroy R: The relationship between weight loss and interleukin 6 in non-small cell lung cancer. *Br J Cancer* **73**, 1560–1562, 1996.

34. Okada S, Okusaka T, Ishii H Kyogoku A, Yoshimori M, et al. : Elevated serum interleukin-6 levels in patients with pancreatic cancer. *Jpn J Clin Oncol* **28**, 12–15, 1998.

35. Kuroda K, Nakashima J, Kanao K, Kikuchi E, Miyajima A, et al.: Interleukin 6 is associated with cachexia in patients with prostate cancer. *Urology* **69**, 113–117, 2007.

36. Bayliss TJ, Smith JT, Schuster M, Dragnev KH, and Rigas JR: A humanized anti-IL-6 antibody (ALD518) in non-small cell lung cancer. *Expert Opin Biol Ther* **11**, 1663–1668, 2011.

37. Zaki MH, Nemeth JA, and Trikha M: CNTO 328, a monoclonal antibody to IL-6, inhibits human tumor-induced cachexia in nude mice. *Int J Cancer* **111**, 592–595, 2004.

38. Soda K, Kawakami M, Kashii K, and Miyata M: Manifestations of cancer cachexia induced by colon 26 adenocarcinoma are not fully ascribable to interleukin-6. *Int J Cancer* **62**, 332–336, 1995.

39. Inui A, Asakawa A, Bowers CY, Mantovani G, Laviano A, et al.: Ghrelin, appetite, and gastric motility: the emerging role of the stomach as an endocrine organ. *FASEB J* **18**, 439–456, 2004.