

The importance of tissue environment surrounding the tumor on the development of cancer cachexia

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Abstract. The relationship between host factors and cancer cachexia was investigated. A single cell clone (clone 5 tumor) established from colon 26 adenocarcinoma by limiting dilution cell cloning methods was employed to eliminate the inoculation site-dependent differences in the composition of cell clones. Clone 5 tumor did not provoke manifestations of cancer cachexia when inoculated in subcutaneous tissue. However, when inoculated in the gastrocnemius muscle, the peritoneal cavity or the thoracic cavity of CD2F1 male mice, typical manifestations of cancer cachexia were observed in all groups of mice with intergroup variations. The blood levels of various cytokines, chemokines and hormones were increased but with wide intergroup variations. Analyses by stepwise multiple regression models revealed that serum interleukin-10 was the most significant factor associated with manifestations of cancer cachexia, suggesting the possible involvement of mechanisms similar to cancer patients suffering cancer cachexia. White blood cells, especially neutrophils, seemed to have some roles on the induction of cancer cachexia, because massive infiltrations and an increase in peripheral blood were observed in cachectic mice bearing clone 5 tumors. The amount of malonyl-CoA in liver correlated with manifestations of cancer cachexia, however the mRNA levels of spermidine/spermine N-1 acetyl transferase (SSAT) (of which overexpression has been shown to provoke manifestations similar to cancer cachexia) were not necessarily associated with cancer cachexia. These data suggest that the induction

of cancer cachexia depends on the environment in which the tumor grows and that the infiltration of host immune cells into the tumor and the resultant increase in inflammation result in the production of cachectic factors, such as cytokines, leading to SSAT activation. Further, multiple factors likely mediate the mechanisms of cancer cachexia. Finally, this animal model was suitable for the investigation of the mechanisms involved in cachexia of cancer patients.

Introduction

Cancer cachexia is a serious problem that affects the majority of the cancer patients (1,2). The characteristics of cancer cachexia are appetite loss, decrease in body weight (BW) decline in storage fat and muscle weights, and deteriorations of nutrition, water, and electrolyte status. In addition, cancer cachexia is associated with poor prognosis, diminished quality of life, and obstacles to cancer treatment (3). Therefore, it is very important to establish a therapeutic strategy for cancer cachexia in order to improve patient quality of life and outcomes. However, the mechanisms underlying cancer cachexia have not been fully elucidated, likely because many factors (including many cytokines and hormones) are involved in the pathogenesis of this condition (4,5).

Some studies have reported that the tumor characteristics play a major role in the pathogenesis of cancer cachexia, while host factors play a minor role (6,7). However, the fact that cancer cachexia develops in the majority of end-stage cancer patients (3,8) but not in patients with certain types of cancer, suggests that the tumor environment may play a larger role. In addition, cachexia-inducing factors, such as various cytokines and hormones (4,5), are produced both by cancer cells and host cells. Using colon 26 murine adenocarcinoma cells, we previously demonstrated that the host's immune function plays an important role in the development of cancer cachexia (9-12).

Tumor cells are comprised of many different kinds of cells. The inoculation of tumor cells provokes a reaction by the host cells, resulting in a selection of tumor cells. Indeed, inoculation of original colon 26 in different sites results in different

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composition of tumor cells, and the selection of cancer cells in response to the host environment results in different clinical manifestations (13). Even when tumor cells are inoculated in nude mice with an immunological deficit, the metastatic capacity of the established tumor varies according to the site of the tumor (14,15).

The present study investigated the relationship between tumor environment and cancer cachexia. In order to exclude the difference of tumor cell type grown in the inoculated site after selection by the immune system, a single cell clone (clone 5 of colon 26 adenocarcinoma) was employed. Clone 5 tumor was established by the limiting dilution method; i.e., it was established from a single cell of colon 26 adenocarcinoma (16).

Materials and methods

Tumor cells. Clone 5 cell, a single-cell clone established from colon 26 adenocarcinoma by the limiting dilution cell cloning method, was employed. Unlike original colon 26, clone 5 does not provoke any symptoms and signs of cancer cachexia in mice when inoculated in the subcutaneous tissues (10,16). Clone 20 cell, another subclone of colon 26, with a potent cachexia-inducing capacity when inoculated in subcutaneous tissues, was also used.

Cells were seeded at a density of 5×10^5 cells/ml in T75 flasks (75 cm², Iwaki Glass, Tokyo, Japan) with RPMI-1640 containing 10% heat-inactivated fetal bovine serum (FBS) (Gibco, Grand Island, NY, USA) and incubated at 37°C in a humidified 5% CO₂ atmosphere until they grew to sub-confluence. They were then trypsinized and re-suspended in Hanks' balanced salt solution (Gibco) at a density of 5×10^5 cells/ml, and 0.2 ml of cell suspension containing 10^6 cells was inoculated either in the subcutis, gastrocnemius muscle, peritoneal cavity, or thoracic cavity.

For determination of cytokine levels in the cell lysate and conditioned medium, 1×10^6 cells in conditioned medium were centrifuged at 500 x g for 5 min at 4°C. The conditioned medium was collected and stored at -80°C until the cytokine assay was performed. The cells were re-suspended with phosphate-buffered saline (PBS). After removal of PBS by centrifugation, the cells were suspended in Procarta lysis buffer (Panomics, Inc., Fremont, CA, USA, from Veritas, Tokyo, Japan) for 5 min on ice. After centrifugation at 19,100 x g for 10 min at 4°C, the supernatant was collected. The supernatants were stored at -80°C until the cytokine assay was performed.

Animals. The experimental design was approved by the Institutional Review Board of Jichi Medical University, and all animal procedures followed the Principles of Laboratory Animal Care. Seven-week-old male CD2F1 (BALB/c x DBA/2 F1) mice were purchased from Charles River Japan (Atsugi, Kanagawa, Japan). Groups of four mice were housed in a cage placed in a temperature-controlled (22°C) room with high efficacy particulate arresting (HEPA; 0.3 µm) filtered air supply and were kept under a 12-h light/12-h dark diurnal cycle. Prior to the experiments, mice were fed *ad libitum* with standard Rodent Laboratory Chow (Nippon Bio-Supply, Tokyo, Japan) so as to become acclimated to experimental conditions. After allowing 1 week of acclimation to the

environment, 8-week-old mice were randomly assigned to one of six different groups: i) mice bearing clone 5 subcutaneously in the right flank (C5 Cut), ii) mice bearing clone 5 in the left gastrocnemius muscle (C5 Mus), iii) mice bearing clone 5 in the peritoneal cavity (C5 Per), iv) mice bearing clone 5 in the thoracic cavity (C5 Tho), v) non-tumor-bearing mice (NTB), and vi) mice bearing clone 20 subcutaneously in the right flank (C20 Cut). Body weight and consumption of food and water were measured every day around 4 p.m.

Blood sampling and extirpation of tumors and organs. Blood was collected from the right atrium of mice under profound pentobarbital (60 µg/g BW) anesthesia at 14 days after tumor inoculation. The right gastrocnemius muscle and epididymal fat pads were extirpated and weighed while wet. Inoculated tumors were extirpated, and 10 mg of tumor was homogenized in 300 µl of Procarta lysis buffer (Panomics) with protease inhibitors and centrifuged at 19,100 x g for 10 min at 4°C. Supernatant was stored at -80°C until the cytokine assay was performed. For histological and immunohistochemical evaluation, extirpated tumors were stored at -80°C until evaluation.

For assessment of blood cell count, 300 µl of the obtained blood was treated with EDTA. Blood cells were counted using a clinical auto-analyzer at a clinical laboratory institute (Japan Pet Life Co., Ltd., Tokyo, Japan). Plasma was obtained from blood treated with heparin. The serum was collected in MiniCollect (Sekisui Chemical Co., Ltd., Tokyo, Japan). Blood samples were centrifuged at 5,000 x g for 10 min at 20°C to separate plasma or serum from blood cells. Plasma and serum were used for the measurement of hormones via a clinical auto-analyzer at a clinical laboratory institute (SRL, Inc., Tokyo, Japan). The serum was stored at -80°C until the cytokine assay was performed.

Serum amyloid A (SAA) was measured by enzyme-linked immunosorbent assay (ELISA) methods as described in a previous report (17,18). A plastic 96-well microtiter plate was coated with the monoclonal antibody diluted in PBS overnight at 37°C and then blocked with 1% bovine serum albumin (BSA) in PBS for 2 h at 37°C. The mouse serum, diluted more than 1:100 in BSA-PBS containing 0.05% Tween-20, was added to the plates and allowed to react for 2 h at 37°C. The plate was washed and then incubated with a peroxidase-conjugated secondary antibody, clone BI-2, diluted in 1% BSA-PBS containing 0.05% Tween-20 for 1 h at 37°C. After a final wash, color was developed in ortho-phenyldiamine and read at 490 nm.

Measurement of cytokine levels. Procarta Cytokine Assay kit (Panomics) was used for cytokine determination. After incubation with antibody-conjugated beads, detection antibodies and streptavidin-phycoerythrin complexes, samples were assessed by a Luminex 100 instrument (Bio-Rad, Hercules, CA, USA). The cytokines examined were as follows: interleukin (IL)-1β, IL-6, IL-10, tumor necrosis factor (TNF)-α, interferon (IFN)-γ, leukemia inhibitory factor (LIF), granulocyte macrophage colony-stimulating factor (GM-CSF), monocyte chemotactic protein (MCP)-1, macrophage inflammatory protein (MIP)-1α and vascular endothelial growth factor A (VEGF-A). A standard curve for sample cytokine concentration determination was used. Cytokine concentrations in tumor homogenates

were corrected relative to protein concentrations measured by the Lowry protein assay.

Real-time PCR. Expressions of spermidine/spermine N-1 acetyl transferase (SSAT) in murine liver were evaluated by real-time PCR. Total RNA was extracted from the homogenized liver tissues using the Illustra RNeasy Mini RNA Isolation kit (GE Healthcare Biosciences, Little Chalfont, UK), and the quality of RNA was assessed using a Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA, USA) according to the manufacturer's instructions. Single-stranded cDNA from total RNA was synthesized using a High Capacity RNA-to-cDNA kit (Applied Biosystems, Carlsbad, CA, USA).

The PCR reactions were performed on ABI PRISM 7900 HT sequence detection system (Applied Biosystems) in a total volume of 50 μ l, containing the appropriate amount of SYBR Premix Ex Taq (Takara Bio, Inc., Otsu, Japan), cDNA template and each primer. Thermal cycling conditions were: 95°C for 10 sec, followed by 40 cycles of 95°C for 5 sec and 60°C for 34 sec. A melt curve was generated after amplification, starting at 95°C for 15 sec, followed by 60°C for 1 min and then 95°C for 15 sec. All reactions were run in duplicate. The Sequence Detection System (SDS2.1) software and the external standard curves were used for analysis. Transcript levels of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were utilized as an internal control. Primers used were as follows: SSAT primers (Invitrogen, Carlsbad, CA, USA): 5'-ATCTAAGCCAGGTTGCAATGA-3' (forward), 5'-GCACTCCTCACTCCTCTGTTG-3' (reverse). GAPDH primers (Invitrogen): 5'-TGTGTCCGTCGTGGATCTGA-3' (forward), 5'-TTGCTGTTGAAGTCGCAGGAG-3' (reverse).

Western blotting. For the measurement of acetyl-CoA carboxylase (ACC), 30 mg of liver in 500 μ l of lysis buffer [RIPA buffer; Cell Signaling Technology, Beverly, MA, USA: 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM Na₂EDTA, 1 mM ethylene glycol tetraacetic acid, 1% nonyl phenoxy-polyethoxyethanol-40, 1% sodium deoxycholate, 2.5 mM sodium pyrophosphate, 1 mM β -glycerophosphate, 1 mM Na₃VO₄, 1 μ g/ml leupeptin] with 1 mM phenylmethylsulfonyl fluoride (PMSF) was homogenized and then sonicated three times for 10 sec on ice. After removing insoluble material by centrifugation at 14,000 x g for 10 min, the total amount of protein was quantified using the Bradford protein assay (Bio-Rad Protein Assay kit; Bio-Rad). Equal amounts of total protein were subjected to SDS-PAGE and then transferred to polyvinylidene difluoride membrane (PVDF) (GE Healthcare Bio-Sciences). The PVDF membrane was blocked with 5% skim milk in Tris-buffered saline with 0.1% Tween-20 (TBS-T) for 3 h at room temperature and then washed two times with TBS-T. The membrane was incubated with anti-acetyl CoA carboxylase antibody (1:200; Cell Signaling Technology) in TBS-T containing 5% BSA and with anti-mouse β -actin antibody (1:3,000; Cell Signaling Technology) in TBS-T containing 5% skim milk at 4°C overnight. The membrane was subsequently rinsed three times and incubated with a horseradish peroxidase (HRP)-conjugated secondary antibody (1:3,000; GE Healthcare) in TBS-T containing 5% skim milk for 1 h and then rinsed three times. Finally, protein bands were detected by enhanced chemiluminescence

(ECL Prime; GE Healthcare) and quantified by densitometry using Image J software.

Malonyl-CoA measurement. Murine livers were weighed and homogenized with a 4-fold weight of 0.3 M H₂SO₄ for 10 min on ice. The supernatants obtained by centrifugation at 10,000 x g for 10 min were adjusted to a pH 6.5 on ice using 1 M Tris 5% v/v.

The measurement of malonyl-CoA was performed as described previously (malonyl-CoA:acetyl-CoA cycling procedures) (19,20). Briefly, acetyl-CoA in tissue extract was removed by 2 μ l of citrate synthase with 100 mM oxaloacetic acid, distilled water and cycling buffer [500 mM Tris-HCl (pH 7.2) containing 100 mM MgSO₄·7H₂O and 10 mM 2-mercaptoethanol]. Then, the cycling reaction was initiated by adding 50 mM malonate with 10 mM ATP and 1.0 U of malonate decarboxylase followed by the addition of 1.0 U of acetate kinase. Next, 2.5 M neutralized hydroxylamine was added, and final concentrations of 10 mM ferric chloride dissolved in 25 mM trichloroacetic acid and 1 M HCl were added. The absorbance of the end product was measured using a spectrophotometer (Ultraspect 3100 Pro, Amersham Biosciences, Buckinghamshire, UK). A standard curve was created using purified malonyl-coenzyme A (Sigma, St. Louis, MO, USA).

Histological and immunohistochemical analysis. Tumor tissues were fixed in 10% formaldehyde and dehydrated in ethanol and xylene, embedded in paraffin and then cut into serial 3- μ m sections. The sections were deparaffinized in xylene, hydrated gradually through graded ethanol and then transferred to PBS. One of the sections was stained with hematoxylin and eosin (H&E), and histological evaluations were conducted by two pathologists and their evaluations were then peer reviewed.

For immunohistochemical analysis, the sections were placed in a container with 10 mM sodium citrate buffer and 0.01% EDTA and heated at 95°C for 30 min for antigenic retrieval. The sections were treated with 1% hydrogen peroxide in methanol to quench the endogenous peroxidase activity for 15 min followed by blocking in 10% normal rabbit serum with 1% BSA in PBS for 2 h at room temperature. The sections were incubated with rat monoclonal anti-neutrophil antibody (1:100; NIMP-R14; Abcam, Cambridge, MA, USA) or rat monoclonal anti-F4/80 antibody (1:100; BM8; Abcam) overnight at 4°C in a humidified chamber. After being washed with PBS, the slides were treated with rabbit anti-rat biotinylated secondary antibodies (1:200; Vector Laboratories, Burlingame, CA, USA), and then an ABC horseradish peroxidase reagent (Vectastain Elite ABC kit; Vector Laboratories) for 30 min at room temperature. Neutrophils and macrophages were visualized using the Peroxidase Substrate kit (ImmPACT DAB; Vector Laboratories) after incubation for 7 min. The sections were lightly counterstained with Mayer's hematoxylin. According to color sampling method (21), a quantitative assay for immunohistochemistry was performed using Image J software. Six fields were examined at x40 magnification and analyzed.

Statistical analysis. Data are expressed as the mean \pm standard deviation (SD). All statistical analyses were performed

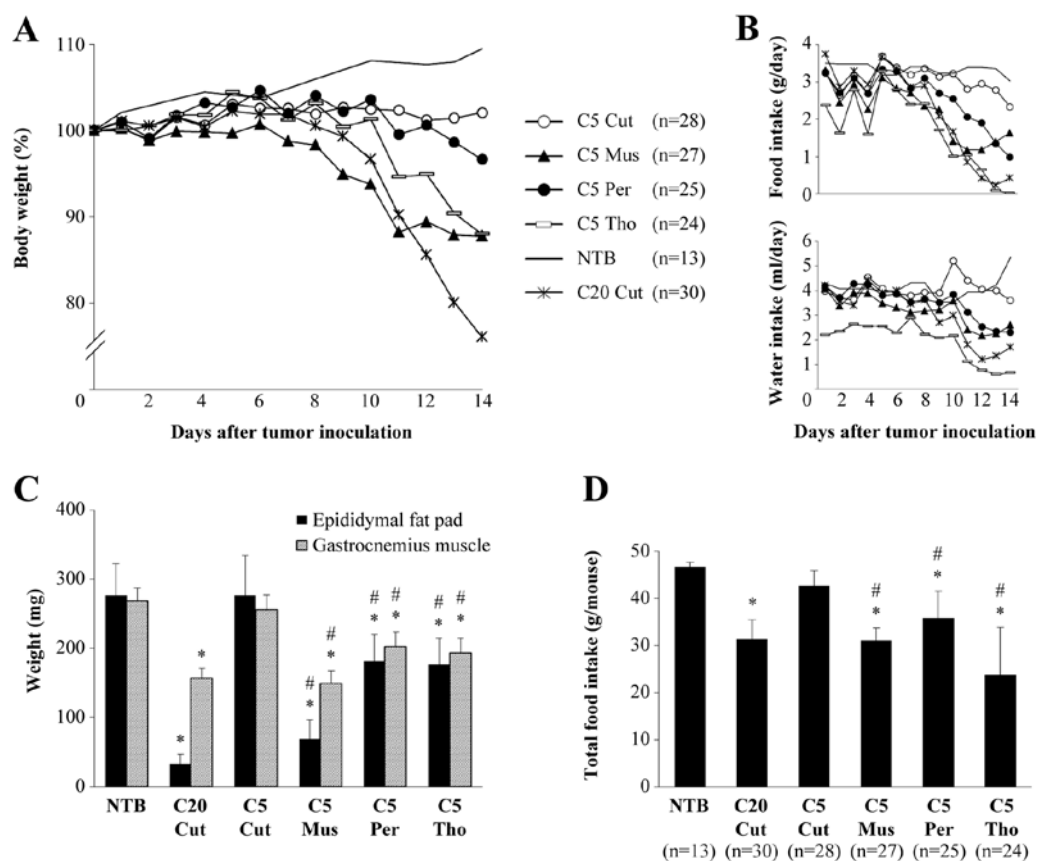


Figure 1. Changes in body weight, food and water intake, and weights of epididymal fat pad and gastrocnemius muscle. (A) Change in body weight. The body weight of NTB and C5 Cut mice did not decrease. Body weight started to decrease around the 9th day after tumor inoculation in C20 Cut, C5 Mus, C5 Per and C5 Tho mice. (B) Change in food and water intake. Mice were housed individually in closed metabolic cages, and food and water intake were calculated daily by subtracting the residues from initial amounts. (C) Weights of epididymal fat pad and gastrocnemius muscle at the end of observation period (n=10). The epididymal fat pad and gastrocnemius muscle were extirpated and weighed at 14 days after tumor inoculation. (D) Total amount of food intake during the observation period. Data represent the mean with bars indicating SD. *Significantly lower than the corresponding value for NTB at $P < 0.05$. #Significantly lower than the corresponding value for C5 Cut at $P < 0.05$. C5 Cut, mice bearing clone 5 tumor in subcutaneous tissue in the right flank; C5 Mus, mice bearing clone 5 tumor in left gastrocnemius muscle; C5 Per, mice bearing clone 5 tumor in peritoneal cavity; C5 Tho, mice bearing clone 5 tumor in thoracic cavity; NTB, control mice bearing no tumor; C20 Cut, mice bearing clone 20 tumor in subcutaneous tissue in the right flank.

using SPSS version 11.0 for Windows (SPSS, Inc., Chicago, IL, USA). Comparisons between two groups of data were analyzed by the Mann-Whitney U test. A correlation between two groups was assessed by the Pearson correlation test. A forward selection stepwise multiple regression model for BW loss and the loss of epididymal fat pad and gastrocnemius muscle was developed using serum cytokines and chemokines with or without hormones as variables. A P-value of < 0.05 was considered to indicate a significant difference.

Results

Body weight, food intake, and fat and muscle tissue weight. Changes in BW, food and water intake, and fat and muscle tissue weight on the 14th day after tumor inoculation and total food intake after tumor inoculation are shown in Fig. 1. BW of mice bearing clone 20 in subcutaneous tissues (C20 Cut) decreased starting at approximately 9 days after tumor inoculation and was 76.1% of those at the time of tumor inoculation. Further, food and water intake significantly decreased. The BW of C5 Cut mice did not decrease, and their amount of food and water intake decreased only slightly. However, the BW and food intake of C5 Mus mice, C5 Per mice, and C5 Tho

mice significantly decreased, albeit with intergroup variation (Fig. 1A and B).

At the end of the observation period, the weights of fat and muscle tissues in C20 Cut were only 12 and 58.3%, respectively, of those in NTB mice, while those in C5 Cut did not decrease. Relative to NTB mice, the weights of the epididymal fat pad and the gastrocnemius muscle were 28.7 and 55.4%, 64.1 and 71.9%, and 65.4 and 75.3% in C5 Mus, C5 Per and C5 Tho, respectively. Among mice bearing the clone 5 tumor, the weights of fat and muscle tissues were the lowest in C5 Mus; further, those in C5 Mus, C5 Per and C5 Tho were significantly lower than those in C5 Cut (Fig. 1C).

Total amount of food intake in C20 Cut was significantly lower than that of NTB. While the amount of food intake did not decrease in C5 Cut, it did significantly decrease in C5 Mus, C5 Per and C5 Tho (Fig. 1D). Although the decreased food intake was accompanied by a decrease in fat and muscle tissue, changes in these variables did not correlate with each other. Although the most prominent decrease in food intake was found in C5 Tho, their fat and muscle tissues were preserved to a greater degree than those of C5 Mus or C20 Cut.

We performed similar experiments using clone 20 cells. Clone 20 cells were inoculated either in muscle tissue,

Table I. Peripheral blood examination.

	WBC (mm ³)	Neutrophils (mm ³)	Monocytes (mm ³)	Lymphocytes (mm ³)	RBC (x10 ⁴ /mm ³)	Hb (g/dl)	Platelets (x10 ⁴ /mm ³)	SAA (mg/l)
NTB	1,571±180	307±55	79±28	1,183±170	946±56	13.5±0.6	81±17	0.4±0.1
C20 Cut	3,514±1229 ^a	1,033±554 ^a	182±93 ^a	2,299±617 ^a	931±34	13.0±0.4 ^a	163±36 ^a	463±142 ^{a,b}
C5 Cut	3,542±315 ^a	861±114 ^a	305±15 ^a	2,497±323 ^a	915±35	13.2±0.5	131±25 ^a	256±178 ^a
C5 Mus	4,928±430 ^{a,b}	1,015±158 ^a	262±67 ^{a,b}	3,644±420 ^{a,b}	905±39	12.6±0.5 ^b	182±10 ^{a,b}	576±104 ^{a,b}
C5 Per	4,628±844 ^{a,b}	1,171±191 ^{a,b}	191±75 ^a	3,258±795 ^{a,b}	899±18 ^a	13.1±0.2 ^a	104±18	64±59 ^{a,b}
C5 Tho	4,757±1429 ^{a,b}	1,290±492 ^{a,b}	206±79 ^a	3,261±722 ^{a,b}	1,061±108 ^{a,b}	15.2±1.4 ^{a,b}	108±7 ^a	28±37 ^{a,b}

Values are mean ± SD (n=7). Blood samples were collected on 14th day after tumor inoculation. ^aSignificantly different from the corresponding value for C20 Cut at P<0.05. ^bSignificantly different from the corresponding value for C5 Cut at P<0.05. C5 Cut, mice bearing clone 5 tumor in subcutaneous tissue in the right flank; C5 Mus, mice bearing clone 5 tumor in left gastrocnemius muscle; C5 Per, mice bearing clone 5 tumor in peritoneal cavity; C5 Tho, mice bearing clone 5 tumor in thoracic cavity; NTB, control mice bearing no tumor; C20 Cut, mice bearing clone 20 tumor in subcutaneous tissue in the right flank; WBC, white blood cells; RBC, red blood cells; Hb, hemoglobin; SAA, serum amyloid A.

Table II. The concentration of serum or plasma hormones.

	Serum (ng/ml)		Plasma (pg/ml)				
	Insulin	Corticosterone	ACTH	Glucagon	Adrenaline	Noradrenaline	Dopamine
NTB	1.4	272.7	114	66	792	1,072	135
C20 Cut	1.3	589.7	88.3	66	699	506	57
C5 Cut	2.7	376.6	269	44	6,149	4,318	179
C5 Mus	2.0	344.8	267	50	2,272	1,411	80
C5 Per	0.7	436.2	205	47	1,933	2,699	262
C5 Tho	1.4	786.4	43.9	65	2,048	4,053	502

Mixture of serum or plasma from six mice was used for measurements. Serum corticosterone was increased in tumor-bearing mice, regardless of the presence or absence of manifestations of cancer cachexia. C5 Cut, mice bearing clone 5 tumor in subcutaneous tissue in the right flank; C5 Mus, mice bearing clone 5 tumor in left gastrocnemius muscle; C5 Per, mice bearing clone 5 tumor in peritoneal cavity; C5 Tho, mice bearing clone 5 tumor in thoracic cavity; NTB, control mice bearing no tumor; C20 Cut, mice bearing clone 20 tumor in subcutaneous tissue in the right flank; ACTH, adrenocorticotrophic hormone.

peritoneal cavity, or thoracic cavity. All of these animals suffered typical cancer cachexia of greater severity and died earlier when compared with C20 Cut.

Blood analyses. When compared with NTB mice, the number of white blood cells (WBC), including neutrophils, monocytes, and lymphocytes, and platelets in the peripheral blood were increased in tumor-bearing mice irrespective of the presence or the severity of cachectic manifestations. No significant difference in peripheral blood examination except for SAA was observed between C20 Cut and C5 Cut. Among C5 groups of mice, the WBC count correlated with the mani-

festations of cancer cachexia. SAA, an acute phase reactant reflecting inflammation in the body, significantly increased in C20 Cut, C5 Cut, and C5 Mus and increase to a lesser degree in C5 Per and C5 Tho, despite the fact that there was a significant increase in the WBC count in C5 Per and C5 Tho mice (Table I).

Corticosterone levels increased in tumor-bearing mice irrespective of the presence of cachectic manifestations. However, levels of other hormones, such as serum insulin, plasma glucagon, and plasma catecholamines, did not correlate with tumor inoculation or the manifestations of cancer cachexia (Table II).

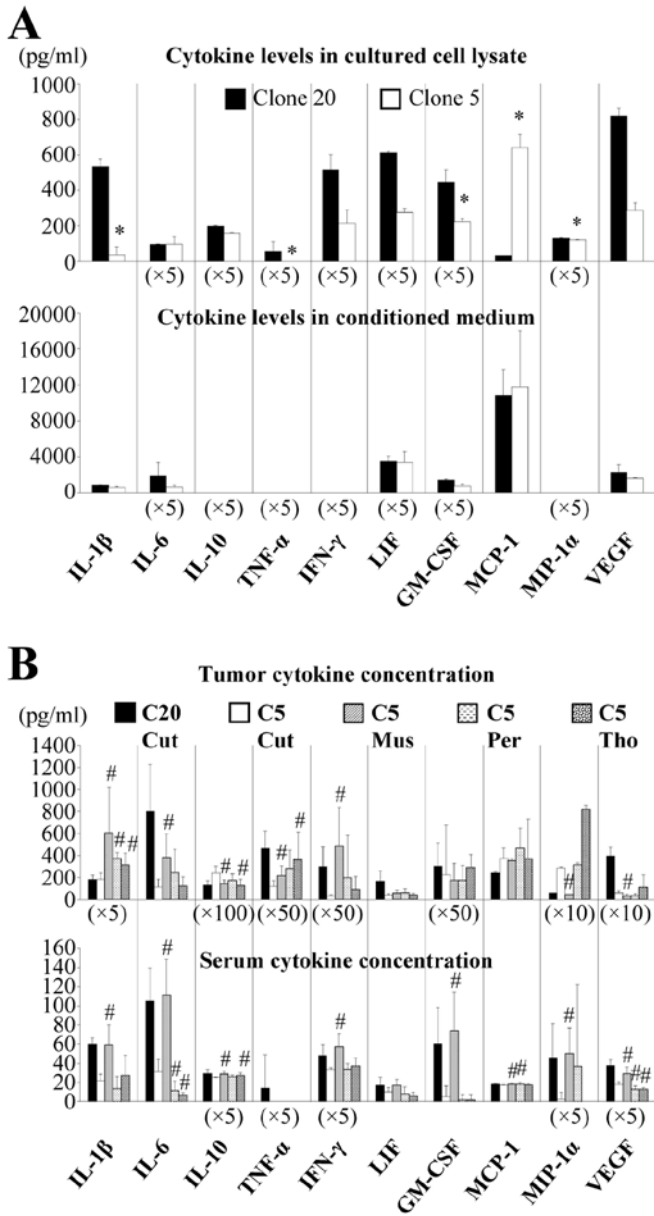


Figure 2. Concentrations of cytokines and chemokines in culture cell lysate, conditioned medium, tumor lysate and murine serum. (A) Cells (1×10^6) ($n=3$) and its conditioned medium ($n=2$) were analyzed. (B) Murine serum and lysates of inoculated tumor ($n=6$). Each value represents the mean with bars indicating SD. *Significantly different from the corresponding value for C20 Cut at $P<0.05$. #Significantly different from the corresponding value for C5 Cut at $P<0.05$.

Cytokine levels. When comparing the levels of various cytokines and chemokines in the conditioned medium, no significant difference was found between clones 5 and 20, despite the fact that the ability of these clones to induce cachexia was significantly different. In cell lysate, levels of the majority of cytokines and chemokines were higher in clone 20 cells than in clone 5 cells, while levels of MCP-1 were lower in clone 20 than in clone 5 (Fig. 2A).

Types and concentrations of cytokines and chemokines in the lysates of the inoculated tumor and in the serum of C5 Cut and C20 Cut groups did not differ significantly when compared with those in conditioned medium and culture cell lysate. Levels of these substances tended to be lower in clone 5 Cut

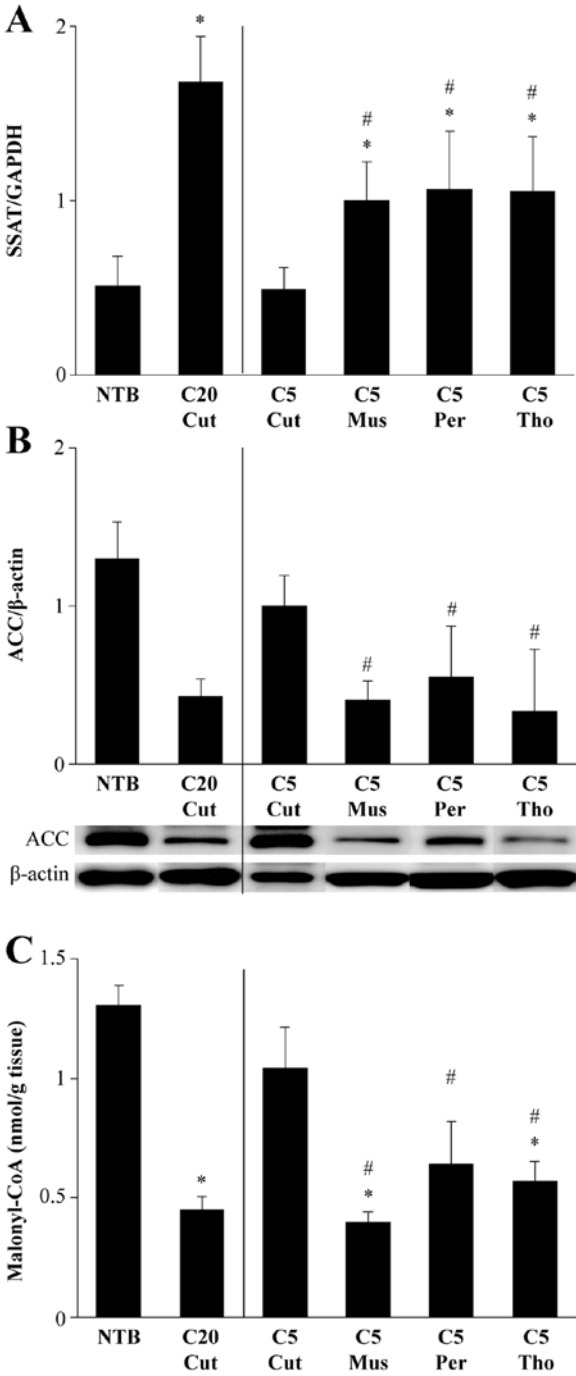


Figure 3. Levels of SSAT mRNA, ACC and malonyl-CoA. Murine liver was extirpated on day 14 after tumor inoculation and examined. (A) SSAT mRNA levels ($n=6$). (B) ACC levels ($n=7$). (C) Malonyl-CoA levels. Malonyl-CoA levels in the liver ($n=6$). Data represent the mean with bars indicating SD. *Significantly different from the corresponding value for C20 Cut at $P<0.05$. #Significantly different from the corresponding value for C5 Cut at $P<0.05$.

than in clone 20 Cut, except for MCP-1 and MIP-1 α . Further, levels of IL-1 β , IL-6, TNF- α and IFN- γ were elevated in tumor lysates of C5 Mus, C5 Per, and C5 Tho when compared with C5 Cut. Although serum IL-1 β , IL-6, IFN- γ , and GM-CSF were increased in C20 Cut and C5 Mus, no such increases were found in C5 Per and C5 Tho (Fig. 2B).

In order to identify factors involved in the induction of cancer cachexia, the mean serum levels of cytokine and chemokines that affect BW and the weights of epididymal fat

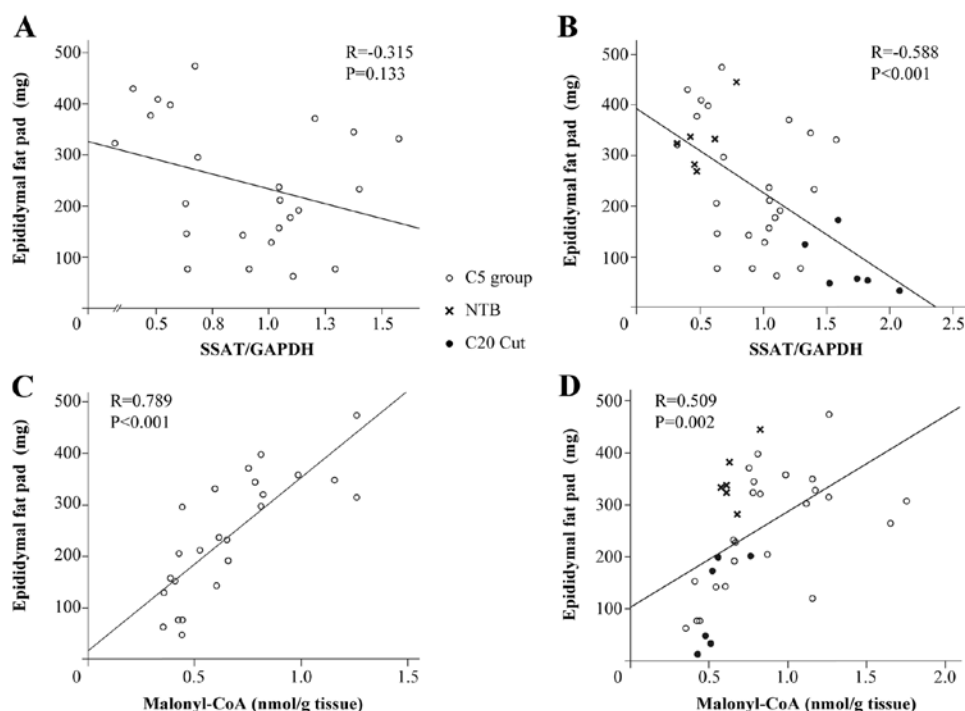


Figure 5. Correlations between epididymal fat weight and SSAT-mRNA levels and between epididymal fat weight and malonyl CoA. (A) Correlations between fat weight and SSAT among mice bearing clone 5 tumor (n=24). (B) Correlations between fat weight and SSAT among all groups of mice, including NTB and C20 Cut (n=36). (C) Correlations between fat weight and malonyl-CoA among mice bearing clone 5 tumor (n=24). (D) Correlations between fat weight and malonyl-CoA among all groups of mice, including NTB and C20 Cut (n=36).

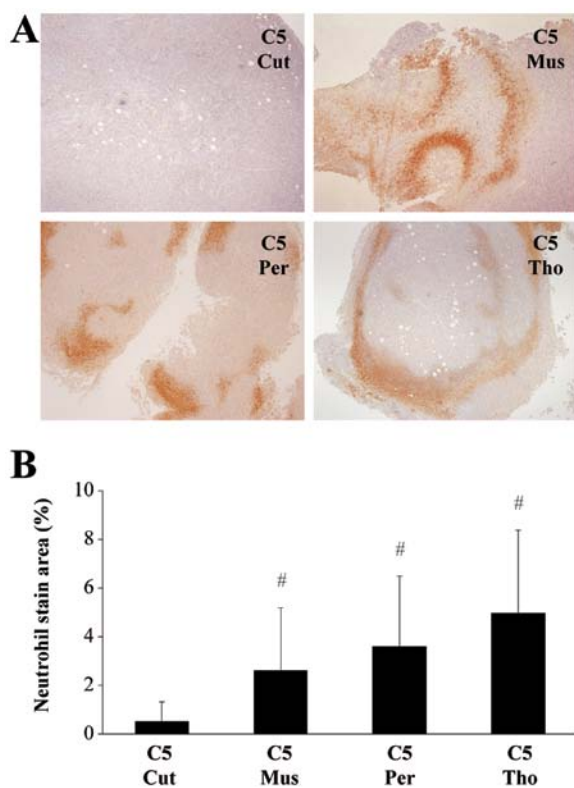


Figure 6. Pathological findings of inoculated tumors. (A) Immunohistochemical findings of neutrophils. There is massive neutrophil infiltration in clone 5 tumors inoculated either in muscle (C5 Mus), the peritoneal cavity (C5 Per), or the thoracic cavity (C5 Tho), while no such findings are present in the tumor in subcutaneous tissue (C5 Cut). (B) The quantitative assay for immunohistochemistry was performed using Image J software (n=6). Data represent the mean with bars indicating SD. #Significantly higher than those of C5 Cut at $P<0.05$.

ence in morphology when comparing tumors from different C5 groups (C5 Cut, C5 Mus, C5 Per, and C5 Tho).

Immunohistochemical examination showed differences in the amount of neutrophils, but not macrophages, among groups of mice bearing the clone 5 tumor. Neutrophils in tumors of C5 Cut and C20 Cut were not significantly increased. However, a significantly large amount of neutrophil infiltration was found in tumors of the C5 Mus, C5 Per, and C5 Tho groups (Fig. 6).

Discussion

The present study showed that host factors are important for the induction of cancer cachexia. Use of the clone 5 tumor, which was established from a single cell in colon 26 adenocarcinoma, obviates the effect of selection of a tumor cell clone by the host's immune system, thereby facilitating experimental investigation of host factors in the present study. Indeed, inoculation of the clone 5 tumor does not result in inoculation site-dependent differences in the composition of cell clones that can otherwise be seen in other animal models where tumors were composed of many cell clones (13-15).

Levels of cytokines and chemokines in the inoculated tumor and levels of cytokines and chemokines in murine serum levels differ significantly depending on the inoculation site. Therefore, manifestations observed in the present animal model can be considered to be due mainly to a difference in the host reaction against the tumor. In addition, because these mice did not have any apparent immune or endocrine defects, the interactions between tumor and host in the present study likely reflected those seen in cancer patients. Indeed, the relationship between cachexia manifestation and serum cytokine

levels among C5 groups was similar to that seen in human cancer cachexia, in which serum IL-10, but not other pro-inflammatory cytokines, is correlated with the manifestations of cancer cachexia (22-24). In the present study, only IL-10, but not IL-1 β , IL-6, or TNF- α , had a negative correlation with BW and the weights of skeletal muscle and storage fat, which contradicts findings from other studies (9,10,25-27).

We could not elucidate a single factor or narrow down a few factors, that play a pivotal role in the induction of cancer cachexia and should therefore be targeted for the treatment of cancer cachexia. However, malonyl-CoA seemed to be a good marker of cancer cachexia, and its preservation might be a therapeutic strategy for cancer cachexia, because the weight of storage fat in mice correlated with the amount of malonyl-CoA in the liver. Malonyl-CoA, converted from acetyl-CoA by ACC, is a substrate of fatty acid synthesis (28). In addition, malonyl-CoA controls the uptake of mitochondrial fatty acid through allosteric inhibition of carnitine palmitoyltransferase 1 (CPT-1), the enzyme catalyzing the first committed step in mitochondrial fatty acid oxidation (29). Therefore, a decrease in malonyl-CoA resulted in decreased fatty acid synthesis and accelerates lipolysis via activation of CPT-1 and ketogenesis (30). The amount of malonyl-CoA is controlled by several factors, such as the amount of its precursor (acetyl-CoA) and the activities of ACC, malonyl-CoA decarboxylase, and SSAT (19,31). SSAT overexpression provokes symptoms similar to cachexia in animals (31), and several cytokines, hormones, natural substances, and stress are known to stimulate SSAT (32-37). The continuous SSAT activation leads to an increased demand for acetyl-CoA (a co-factor of SSAT), thereby restricting conversion of acetyl-CoA to malonyl-CoA. However, among mice bearing the clone 5 tumor, SSAT mRNA levels did not correlate with the severity of cachectic manifestations. These observations suggest that some other factors that lead to a decrease in malonyl CoA levels likely play a role in the induction of cancer cachexia.

Recent clinical reports suggest that an increase in neutrophils and neutrophil infiltration to the tumor are associated with poor outcomes and an increasing severity of cachexia manifestations (38-42). In animal models, although circulating GM-CSF levels were not necessarily increased in cachectic mice, an increase in peripheral blood WBC and neutrophils correlated with cachectic manifestations of mice bearing the clone 5 tumor. The involvement of neutrophil is supported by immunohistochemical findings, because neutrophil infiltration was abundant in clone 5 tumors of mice suffering from cancer cachexia. We previously demonstrated that the suppression of granulocyte differentiation by the enhancement of erythrocyte production results in an attenuation of the manifestations of cancer cachexia (12). However, the grade of neutrophil infiltration was not necessarily accompanied by a change in SAA levels (as an indicator of systemic inflammation) or a change in the serum levels of cytokines and chemokines. These must be dependent on the specific environment of tissues and organs in which the tumor grows.

In conclusion, the present study suggests that host factors and host-tumor interactions are very important for the induction of cancer cachexia. Further, liver malonyl-CoA likely plays an important role in cancer cachexia. Finally, the animal model used in the present study appears to be suitable for

the investigation of the mechanisms involved in cachexia of cancer patients.

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