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Characterization of the Yoshida Sarcoma: A Model of Cancer Cachexia

Mary Ann Honors¹ and Kimberly P. Kinzig¹

¹Department of Psychological Sciences and Ingestive Behavior Research Center, Purdue University, 703 Third Street West Lafayette, IN 47907

Abstract

Purpose—Cancer cachexia contributes significantly to morbidity and mortality in individuals with cancer. Currently, the mechanisms contributing to the development of cachexia are largely unknown, leading to a paucity of treatment and prevention options. Animal models are necessary in determining causal mechanisms and in testing potential treatments. While the Yoshida sarcoma has been utilized for 50 more than years, the cachexia syndrome produced by this model has not been well characterized in the literature.

Methods—Tumor allografts were subcutaneously implanted in male Sprague Dawley rats (n = 16), and allowed to grow for 23 days. Control animals (n = 16) received a sham surgery. All rats were monitored daily for the presence of hallmark cachexia symptoms.

Results—The present results demonstrate the presence of decreased body weight gain, as well as lower levels of body adiposity and skeletal muscle mass, in tumor-bearing animals, as compared to controls.

Conclusions—While a large tumor burden was reached, the extent of cachexia was similar to that which is observed in many individuals with cancer cachexia. Future experiments utilizing this model are encouraged to identify mechanisms and effective treatment and prevention strategies.

Keywords

cancer cachexia; animals models; Yoshida sarcoma

Introduction

Cancer cachexia, a syndrome of weight loss, loss of appetite, and wasting of body tissues, contributes significantly to both morbidity and mortality in individuals with cancer [1]. Individuals who experience cachexia have reduced survival time, as well as diminished psychological and physical health [2,3]. In addition, cachectic cancer patients often display more negative side effects and a decreased therapeutic response during chemotherapy [4–6]. The majority of individuals with cancer will experience some degree of cachexia during the course of their disease [7], making cancer cachexia a clinically relevant syndrome for which the causal mechanisms are largely unknown.

Corresponding Author: Mary Ann Honors, 703 Third Street, West Lafayette, IN 47907, USA; phone: (765) 496-1042; fax: 765-496-1264; mhonors@purdue.edu.

Conflict of Interest

The authors have no conflicts of interest to declare. The authors are in possession of the primary data and will allow the journal to review the data, if requested.

Limited evidence-based therapies are available for the prevention and treatment of cancer cachexia. In order to develop such therapies, proper animal models are necessary. To yield the best translational research, models must produce symptoms to a similar degree as those that are experienced by individuals with cancer. The Yoshida sarcoma is one transplantable allograft tumor model utilized in the study of cancer and cancer cachexia [8–17]. While the Yoshida sarcoma has been utilized for 50 years, a proper detailed characterization of the development of cachexia symptoms in this model, as well as the tumor growth pattern, is lacking in the scientific literature.

The present experiment was designed to characterize the progression of cancer cachexia symptoms in male Sprague Dawley rats bearing the Yoshida sarcoma. In addition, the pattern of growth of the subcutaneous tumors was examined. The results demonstrate that the Yoshida sarcoma is an effective model of cancer cachexia, and should be utilized in future experiments.

Methods

Experimental Animals

Thirty two male Sprague Dawley rats (Harlan Sprague Dawley, Indianapolis, IN) served as experimental subjects. Rats were individually housed in hanging wire mesh cages in a temperature and humidity controlled room, with a 12:12h light:dark cycle (lights on at 04:00). Following arrival in the laboratory, rats were allowed to acclimate to the laboratory environment for one week prior to the start of the experiment. Throughout the experiment, all rats received *ad libitum* access to tap water and semi-purified rodent chow (AIN-76A, Dyets Inc., Bethlehem, PA). All procedures were approved by the Purdue University Animal Care and Use Committee.

After acclimation to the laboratory environment, rats were weight-matched and divided into two experimental groups ($n = 16$ per group), which differed in tumor status. The control group received a sham surgery. The tumor-bearing group (TB) was subcutaneously implanted with a Yoshida sarcoma allograft. Surgery was performed on experimental Day 0. Animals were weighed daily, and food intake, minus food spillage, was measured and recorded.

Tumors and Tumor Implantation

Yoshida sarcoma ascites tumor cells were purchased from the Development Therapeutics Program at the National Cancer Institute (Bethesda, MD). Donor animals were utilized to perpetuate the tumor line in the laboratory for the duration of the experiment. The original donor animal received a subcutaneous injection of approximately 4×10^6 cells into the right flank and a solid tumor soon developed.

Tumor tissue was dissected from the donor rat for implantation into experimental tumor-bearing animals. The donor rat was overdosed with sodium pentobarbital and the area surrounding the tumor was sterilized with betadine solution. The tumor tissue was then removed, cleaned of any visible necrotic tissue, divided into fragments, and placed on ice. The animal receiving the tumor was anesthetized under isoflurane anesthesia and the right flank was shaved and sterilized. An incision was then made in the skin and a small subcutaneous pocket was created. The tumor fragment (approximately 6 mm^3) was placed in the pocket and the incision was closed with sterile silk suture. All animals for the experiment received tumor tissue from the same donor animal. Sham surgeries were performed in control animals.

Tumor volume was determined through measurement of tumor size by external digital caliper, with the longest longitudinal diameter (length, L), greatest transverse diameter (width, W), and the great vertical diameter (height, H) recorded daily. Tumor volume was calculated using a standard ellipsoid formula ($V = \pi/6 \times (L)(W)(H)$) [18]. A linear regression equation for the relationship between tumor volume and tumor weight was generated based on all tumor-bearing animals in the laboratory to date, and was used to calculate approximate tumor weight throughout the experiment.

Measurement of Body Composition

Body composition was measured on Days 6, 13, and 20. Fat mass was analyzed in conscious rats using the EchoMRI system (Echo Medical Systems, Houston, TX). Data are presented as a percentage of body fat. Hind leg diameter was utilized as a measure of skeletal muscle mass. The contralateral hind leg, relative to the tumor, was extended and the diameter of the upper leg was measured via external digital caliper and recorded.

Sacrifice and Terminal Measures

Rats were sacrificed on Day 23 under ether inhalation anesthesia followed by rapid decapitation approximately 4 hours prior to the start of the dark cycle. Trunk blood was collected in for analysis of non-fasting blood glucose and plasma insulin levels. Blood glucose levels were measured using the Precision Xtra Glucose Monitoring System (Abbott Laboratories, Abbott, IL). The remaining blood samples were centrifuged at 2000 rpm for 15 minutes at 4°C, and plasma was removed and stored at -80°C for subsequent analysis of plasma insulin levels by radioimmunoassay.

The epididymal fat pads were removed from each animal and weighed, as a measure of terminal fat mass. Additionally, hind leg diameter, both contralateral and ipsilateral to the tumor, was recorded, as a measure of terminal skeletal muscle mass. In TB rats, the tumor was also removed and weighed. Small samples of epididymal fat and quadriceps muscle were collected and placed in RNAlater for analysis of mRNA expression by QPCR.

Radioimmunoassay (RIA)

Plasma insulin levels were determined using a commercial Rat Insulin RIA kit (Millipore, Billerica, MA), with upper and lower detection limits of 0.1 ng/mL and 10 ng/mL, respectively. All samples were run in duplicate and per manufacturer's instructions. Unknown concentrations of insulin were calculated based on a standard curve generated for each kit.

Quantitative Real-Time Polymerase Chain Reaction (QPCR)

RNA was isolated from tissue samples for determination of mRNA expression. Tissue samples (100mg) were homogenized in 2 mL of Trizol Reagent (Invitrogen, Carlsbad, CA), according to the manufacturer's protocol. cDNA was synthesized from 0.5–3µg of RNA using the SuperScript III First-Strand Synthesis System (Invitrogen, Carlsbad, CA) and diluted in nuclease-free water for storage at -80°C. Primers included L32 (Forward: 5'-CAGACGCACCA TCGAAGT TA-3'; Reverse: 5'-AGCCACAAAGGACGTGTTTC-3'), -actin (Forward: 5'-CGTGGGCCGCCCTAGGCACCA-3'; Reverse: 5'-CTCTTTTGATGTACGCACGATTTC-3'), Atrogin-1 (Forward: 5'-GTCCAGAGAGTCGGAAGTC-3'; Reverse: 5'-GTCGGTGAT CGTGAGACCTT-3'), and Hormone Sensitive Lipase (HSL) (Forward: 5'-GAATATCACGGA GATCGAGG-3'; Reverse: 5'-CCGAAGGGACACGGTGATGC-3'). QPCR was performed in duplicate using a BioRad iCycler and Maxima Sybr Green solution (Thermo Scientific, Barrington, IL) with two-step (L32 and Atrogin-1) or three-step (-actin and HSL) amplification for 40 cycles.

L32 was amplified from each sample for use as an endogenous control for Atrogin-1, while β -actin served as an endogenous control for HSL. The method of Pfaffl was used to determine expression of the genes of interest [19]. Expression was calculated based on normalization to the housekeeping gene, relative to the efficiency of the housekeeping and interest genes in the target tissue.

Statistical Analyses

Data are presented as mean $\pm$ standard error of the mean. All statistical analyses were performed using GraphPad Prism Software.

The tumor growth curve was analyzed for fit in an exponential equation, with linear growth as an alternative model. A linear regression equation was generated by comparing tumor volume and tumor weight in all experimental animals generated to date, and utilized to calculate the approximate weight of each TB rat's tumor during the experiment. Pearson R correlation analysis was performed to examine the relationship between calculated final tumor weight and actual final tumor weight.

Total weight and body weight (total weight minus approximate tumor weight) were analyzed via two-way ANOVA with tumor status (control or tumor-bearing) and time as independent variables. Planned Bonferroni comparisons were performed when appropriate. Mean daily food intake was calculated for each group and analyzed by Student's T-test. Body composition data for each time point were analyzed separately via Student's T-tests, as were terminal measures of body composition, and blood glucose and plasma insulin levels.

Data relating to mRNA expression in each tissue, as measured by QPCR, were also analyzed via Student's t-test.

Results

Total Weight and Body Weight

Total weight was measured daily, and adjusted for approximate tumor weight at the end of the experiment (Figure 1). Analysis of total weight (body weight plus tumor weight) by two-way ANOVA revealed significant main effects of tumor status ($F(1, 720) = 4.065, p < 0.05$) and time ($F(23, 720) = 49.39, p < 0.0001$), as well as a significant interaction ($F(23, 720) = 3.618, p < 0.0001$). Planned Bonferroni comparisons demonstrated that total body weight was significantly higher in TB animals on Day 20, 22, and 23 ($p < 0.05$ for each) (Figure 1A). Because the tumor burden contributed significantly to the total weight of each animal, body weight was calculated by subtracting approximate tumor weight from total weight at each time point. Analysis of body weight by two-way ANOVA revealed a significant main effects of tumor status ($F(1, 720) = 13.94, p < 0.0001$) and time ($F(23, 720) = 20.99, p < 0.0001$), as well as a significant interaction between these two variables ($F(1, 23) = 7.86, p < 0.0001$). Planned Bonferroni comparisons revealed body weight was significantly lower in TB animals, as compared to controls, starting on Day 16 and continuing to the end of the experiment on Day 23 ($p < 0.01$ for Day 16–23) (Figure 1B).

Food Intake

When average daily food intake was calculated for each group across the whole experimental time period, it was observed that TB rats consumed significantly less food than control rats (Control: $20.56 \pm 0.15\text{g}$; TB: $19.58 \pm 0.18\text{g}$, $t(542) = 4.311, p < 0.0001$), a difference of approximately 1 gram per day (mean difference = $0.9857 \pm 0.2286\text{g}$).

Tumor Volume and Tumor Weight

Tumor volume was monitored daily in tumor-bearing animals, with 100% of animals within this group developing a tumor. No tumors developed in any control animals. Tumors followed an exponential growth curve, with a doubling time of approximately 4.1 days (Figure 2A). The regression equation generated from all tumors produced in the laboratory for the purpose of determining approximate tumor weight was $y = 0.998x + 2.4173$, where x is tumor volume and y is tumor weight (Figure 2B). Tumor volume and weight for all tumors were significantly correlated ($R = 0.9500$, $p < 0.0001$) (Figure 2B). When approximate tumor weight was calculated based on the regression equation, the curve for calculated tumor weight followed an exponential growth pattern (Figure 2C). For tumors in this experiment, actual final tumor weight was significantly correlated with calculated final tumor weight ($R = 0.6497$; $p < 0.01$) (Figure 2D).

Body Composition

On Day 6, Student's t-tests revealed no significant differences in body composition in tumor-bearing animals, as compared to control animals. Percent body fat and hind leg diameter were comparable in both control and tumor bearing groups ($t(14) = 1.104$, $p > 0.05$, and $t(14) = 1.059$, $p > 0.05$, respectively) (Figure 3A and 4A, respectively). On Day 13, Student's t-test identified an interesting pattern of body composition in tumor-bearing animals. At this time point, tumor-bearing animals exhibited a lower percent body fat, as compared to control animals ($t(14) = 2.456$, $p < 0.05$) (Figure 3B), while exhibiting no difference in hind leg diameter ($t(14) = 0.1452$, $p > 0.05$) (Figure 4B). On Day 20, tumor-bearing rats exhibited a significantly lower percent body fat ($t(14) = 3.168$, $p < 0.01$) and a smaller hind leg diameter ($t(12) = 2.672$, $p < 0.05$), as compared to control rats (Figure 3C and 4C, respectively).

On the day of sacrifice (Day 23), terminal measures of body composition supported the group differences in body composition observed on Day 20. Epididymal fat pad weight was significantly lower in tumor-bearing rats, as compared to controls ($t(30) = 2.679$, $p < 0.05$) (Figure 5A). Similarly, hind leg diameter was significantly smaller in tumor-bearing rats on both the contralateral ($t(30) = 6.758$, $p < 0.0001$) (Figure 5B) and ipsilateral side ($t(30) = 3.609$, $p < 0.01$) (Figure 5C), relative to the tumor, at the time of sacrifice.

Terminal Blood Glucose and Plasma Insulin

Analysis of terminal levels of blood glucose did not reveal a significant difference between non-fasting glucose levels in TB and control animals (Control: 153.7 ± 5.63 mg/dL; TB: 148.3 ± 8.37 mg/dL, $t(30) = 0.5329$, $p > 0.05$). However, plasma insulin concentrations were significantly lower in tumor-bearing rats, as compared to controls, according to Student's t-test (Control: 2.90 ± 0.27 ng/mL; TB: 1.00 ± 0.18 ng/mL, $t(28) = 2.799$, $p < 0.05$).

QPCR Analysis

Expression of Atrogin-1 was significantly higher in skeletal muscle samples of tumor-bearing rats, as compared to controls ($t(9) = 3.738$, $p < 0.01$) (Figure 6A). HSL expression in epididymal fat samples was not significantly different between groups (Figure 6B).

Discussion

Animal models are essential for the development of treatment and prevention strategies for individuals with cancer cachexia. While the Yoshida sarcoma has been utilized in cancer research for nearly 50 years [9], the progression of cachexia symptoms in this model has not been well characterized in the literature. In the present experiment, male Sprague Dawley

rats implanted with the Yoshida sarcoma developed the cachexia syndrome and prove to be a useful model for the study of cancer cachexia.

When implanted subcutaneously, the Yoshida sarcoma had a 100% success rate in the experiment. All rats implanted with tumor fragments subsequently developed a tumor, while no control animals developed a tumor. The tumors were not metastatic, despite a tendency to invade the abdominal wall. In contrast, many other cancer cachexia models result in metastatic tumors [20]. The presence of metastatic tumors significantly reduces the ability to explore the causal factors of cancer cachexia development, as the infiltration of tumors into distant organs can have wide ranging effects, depending on the location or extent of the metastatic spread. For this reason the non-metastatic Yoshida sarcoma model is an ideal choice for such mechanistic studies.

While body weight was significantly lower in tumor-bearing animals, a minimal decrease in food intake was observed in tumor-bearing rats in the present experiment. While statistically significant, the practical significance of this approximately 1 gram per day difference is questionable. Considering the small difference in food intake and the more substantial difference in body weight, we conclude that alterations in food intake have minimal significance in producing cachexia in this model. In contrast, multiple models of cancer cachexia produce profound anorexia [20], making it difficult to determine if the changes in body weight and body composition are due to differences in food intake or the actual development of cachexia. The Yoshida sarcoma model does not produce a meaningful difference in daily food intake, allowing this factor to be ruled out as an influence on cachexia development.

Tumor-induced changes in body weight were accompanied by lower levels of fat and muscle mass. Percent body fat was significantly lower in tumor-bearing animals on Day 14, while skeletal muscle mass was only significantly lower on Day 21. While it is possible that muscle mass was reduced prior to Day 21 but after Day 14, our results indicate that changes in body fat occur prior to changes in muscle mass in this model of cancer cachexia. In agreement with these observations, previous research suggests that the mobilization of fatty acids may occur prior to the onset of weight loss [21], indicating that alterations in lipid metabolism are an important early event in the development of cachexia. Further, fat mass is typically lost more quickly than lean mass in individuals with cachexia [22]. Current methods for the assessment of body composition, however, need to be improved, and the continued monitoring of body composition changes during cancer cachexia as these improvements are made.

In agreement with the body composition data, QPCR analysis of gene expression revealed increased expression of Atrogin-1 in skeletal muscle. Atrogin-1 is an important enzyme in the ubiquitin-proteasome pathway for muscle proteolysis, and is highly expressed in animal models of cachexia [23]. While not directly measured in this experiment, this indicates the presence of increased protein degradation within muscle and is congruent with previous research in this model demonstrating a reduction in total body protein content [14]. These data also demonstrate that muscle wasting occurs within this model, as an increase in Atrogin-1 expression would not be observed if muscle growth was simply retarded without actual muscle degradation and wasting.

In contrast, QPCR analysis of hormone-sensitive lipase mRNA expression in epididymal fat samples did not reveal a significant difference between groups. Hormone sensitive lipase is one important driver of lipolysis [24,25] and its expression is increased in individuals with cancer cachexia [26]. It is possible that other fat depots (subcutaneous, retroperitoneal) may have experienced a different pattern of mRNA expression, as fat depots may be regulated

independently. However, while HSL activity is regulated on a transcriptional level, Reynisdottir and colleagues have previously observed no correlation between HSL mRNA levels and HSL activity or lipolysis rate in healthy human adipose tissue samples [27]. This suggests that HSL activity may be altered in tumor-bearing animals, despite no change in mRNA expression. Future experiments should examine HSL activity in epididymal fat, as well as mRNA expression and HSL activity in other fat depots.

Our results demonstrate the presence of cancer cachexia in Yoshida sarcoma-bearing rats. Despite a large tumor burden, the degree of cachexia produced by this model is similar to that which is observed in individuals with cancer. On the day of sacrifice, tumor-bearing animals weighed approximately 11% less than controls. A 10–20% weight loss common in individuals with cancer cachexia [28,2]. Mean daily food intake was approximately 5% less in tumor-bearing animals, which is, in fact, a smaller difference than is typically observed in individuals with cachexia [29]. In addition, terminal hind leg diameter was approximately 7% lower and epididymal fat pad weight was approximately 15% lower in tumor-bearing rats, indicating a significant decline in both fat and muscle mass.

In the present experiments, tumor-bearing animals developed full cancer cachexia symptoms in approximately 21 days. This relatively short time period allows for careful evaluation of the development of cachexia symptoms, and also provides a duration that is long enough to allow for the testing of preventative agents. In contrast, many animal models require either very few days or multiple months to produce cachexia symptoms [20]. One limitation of this model, however, is the large tumor volume, which could be prohibitive for longer term studies.

While no animal model perfectly reflects the human condition, the extent to which the Yoshida sarcoma model mimics human cachexia is remarkable. Overall, the use of this model for investigating the development of and prevention of cancer cachexia is encouraged.

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Fig 1. Total and body weight measures in control and tumor-bearing (TB) rats. (A) Total weight was significantly higher in TB rats, as compared to controls, on Day 20, 22, and 23. (B) Body weight (total weight minus approximate tumor weight) was significantly lower in TB rats, as compared to control rats, on Day 16–23. * $p < 0.01$

Fig 2.

Tumor growth following implantation of Yoshida sarcoma allografts in TB rats. (A) Tumor volume was measured daily and calculated using a standard ellipsoid formula. Tumor volume followed an exponential growth pattern. (B) Final tumor volume and final tumor weight for all tumors generated in the laboratory were utilized to calculate a regression equation for use in calculating approximate tumor weight during the tumor growth period in experimental rats. (C) Calculated tumor weight in experimental animals, based on the regression formula. Calculated tumor weight also followed an exponential pattern. (D) Calculated final tumor weight and actual final tumor weight were significantly correlated in experimental animals

Fig 3. Body adiposity in control and tumor-bearing (TB) rats, as measured by EchoMRI. While control and TB animals had a similar percent body fat on Day 6 (A), TB animals had significantly less body fat than controls on Day 13 (B) and Day 20 (C). * $p < 0.05$

Fig 4. Contralateral hind leg diameter in control and tumor-bearing (TB) rats. While control and TB animals had a similar contralateral hind leg diameter on Day 6 (A) and Day 13 (B), TB animals had significantly smaller hind leg diameter than controls on Day 20 (C). * $p < 0.05$

Fig 5.

Body composition data collected on the day of sacrifice (Day 23) in control and tumor-bearing (TB) rats. (A) Epididymal fat pad weight was significantly lower in TB rats, as compared to chow ($p < 0.05$). Contralateral (B) and Ipsilateral (C) hind leg diameter were also lower in TB rats, as compared to chow. * $p < 0.05$

Fig 6. mRNA expression, as measured by QPCR, in (A) quadriceps muscle, (B) epididymal fat samples. Atrogin-1 mRNA expression was significantly higher in quadriceps muscle samples TB rats, as compared to controls. No differences in HSL expression were observed. *p < 0.05