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ORIGINAL ARTICLE

IMMUNOCOMPETENT MURINE MODEL OF CANCER CACHEXIA FOR HEAD AND NECK SQUAMOUS CELL CARCINOMA

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Abstract: *Background.* Muscle wasting and weight loss were observed when carcinomas were induced in a murine model of head and neck squamous cell carcinomas. Our hypothesis was C3H/HeN mice would develop evidence of cachexia when injected with tumor cells.

Methods. Age- and weight-matched female mice were injected with SCCF/VII cells. Daily food intake and weights were measured. Body composition and analysis of circulating cytokines was performed. At the completion of experiments, hind legs were weighed. Muscle atrophy was detected using analysis for muscle ring finger 1 (MuRF1). The tumor-derived lipid mobilizing factor (LMF) was measured.

Results. Despite increased food intake, tumor-bearing mice lost weight and experienced reduced hind leg weights. Interleukin-1β (IL-1β) was increased and MuRF1 was present in tumor-bearing mice but not controls. LMF was present in SCCF/VII cells.

Conclusion. In this immunocompetent murine model, we demonstrated the development of cancer cachexia in mice inoculated with SCCF cells, which express LMF. There was increased serum IL-1β, weight loss, and muscle wasting and atrophy in tumor-bearing mice.   © 2007 Wiley Periodicals, Inc. *Head Neck* **30:** 320–326, 2008

Keywords: cancer cachexia; head and neck cancer; muscle atrophy

Cachexia is a catabolic state characterized by a loss of lean body mass and fat mass. It remains a major cause of morbidity and mortality for patients with cancer.[1] Loss of muscle mass and unintentional weight loss cause generalized weakness,

emotional and mental fatigue, and an overall general decline in health. Cancer-induced weight loss has been shown to adversely affect prognosis and quality-of-life for patients.[2] In addition, this catabolic process impairs response to chemotherapy and results in increased treatment-related complications and toxicities.[3] Cancer survival has been shown to be directly related to both the rate and degree of weight loss.[3] Cachexia has been estimated to account for up to 20% of all cancer deaths.[4,5] Therefore, patients with head and neck cancer remain at particular risk for cachexia.[6,7]

Although the molecular basis for cachexia remains to be fully elucidated, several mediators have been identified and can be categorized into 2 classes: tumor-derived factors and host-derived factors. A well-known tumor-derived factor called lipid mobilizing factor (LMF) has been shown to induce cachexia in existing animal models. In cachectic mice bearing murine adenocarcinoma (MAC16) tumors, loss of adipose tissue is associated with increased LMF expression in white adipose tissue.[8] This protein is also called zinc-alpha(2)-glycoprotein (ZAG).[8,9] This protein appears to play a pivotal role in the loss of adipose tissue seen in cancer cachexia.

There is also compelling evidence that proinflammatory cytokines play a causative role in the onset and progression of cachexia. Particular interest has focused on interleukin-1-beta (IL-1β), tumor necrosis factor alpha (TNF-α), interferon-gamma (IFN-γ), and interleukin-6 (IL-6) due to their ability to cause muscle wasting and potentiate cachexia.[10,11] Skeletal muscle protein degradation appears to be regulated by the ubiquitin proteasome pathway. Two predominant muscle-specific E3 ubiquitin ligases, muscle ring finger 1 (MuRF1) and atrogin-1/muscle atrophy Fbox (MAFbx), are selectively elevated in skeletal muscle catabolism.[12]

To date, only a few transplantable tumors are capable of inducing cachexia in vivo in animals.[13] Current animal models of cachexia utilize only a few cell lines, and a model for head and neck cancer cachexia currently does not exist. Athymic mice bearing human tumor xenografts are limited by the inability to study the role of host immune system as a contributory factor in fat and muscle loss. Insights from a new immunocompetent animal model can allow us to study tumor contributions to cancer cachexia and to better elucidate tumor–host immune interaction. An immunocompetent model of cachexia will also allow for screening of potential pharmacological agents to use in the treatment of cancer and of cachexia. Based upon observations of wasting seen in an established murine model of head and neck cancer, we hypothesized that cancer cachexia could be induced in a reproducible way by injecting a squamous cell carcinoma cell line (SCCF/VII cells) into immunocompetent C3H/HeN mice. We investigated whether this model of cancer cachexia recapitulates the features of human head and neck cancer cachexia by examining body weight, body composition, circulating cytokine levels, and presence of MuRF1 and LMF.

MATERIALS AND METHODS

Cell Culture and Reagents. The SCCF/VII (SCCF) cell line is a squamous cell carcinoma line that mimics oral carcinoma after being injected into syngeneic C3H/HeN mice.[14,15] Tumor cells were propagated in tissue culture flasks at 37°C, with 5% CO_2 in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. SCCF cells were grown in culture to 80% confluency, and then harvested, washed, and resuspended in phosphate-buffered saline (PBS). On day 0, mice ($n = 5$) were inoculated subcutaneously (s.c.) in bilateral flanks with 1×10^5 cells in 100 μL volume. Age- and weight-matched control mice ($n = 5$) received a sham injection of PBS. Mice were injected in the flanks to avoid any interference with oral food intake.

Mice. Six- to eight-week-old female immunocompetent C3H/HeN mice weighing 21 to 22 g were purchased from Charles River Laboratory (Wilmington, MA). Animals were housed at the University of North Carolina animal facility in individual plastic cages (1 mouse per cage) in a temperature- and humidity-controlled room with a 12-hour dark/light cycle. Animals were fed a defined AIN-93M diet (Granville Milling; Creedmoor, NC) and received tap water ad libitum. All animal procedures were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of North Carolina at Chapel Hill. Mice were allowed to grow tumors for 22 or 23 days, when wasting was apparent and the tumor burden had reached the accepted limit set by the Department of Laboratory Medicine and IACUC. The animals were then euthanized.

Assessment of Food Intake, Body Weight, Tumor Size. In 1 set of experiments, animals were housed in individual cages for accurate measurement of

food consumption. The amount of ingested food was measured in all cages by taking daily weights of food (in grams). Every 2 days the body weight and tumor volume of all mice were measured. Tumor growth was assessed using calipers in 2 dimensions. The maximal orthogonal diameter of the tumor was determined using the formula: mean diameter $= (A + B)/2$. The gross size and weight of the tumors were determined after harvesting the tumors from euthanized animals at the completion of the study. Final body weight was then calculated by subtracting tumor weight (obtained by resection) from total weight.

To determine differences in mass of quadriceps and gastrocnemius muscles, mouse hind legs were weighed immediately after euthanasia. The weights did not include any tumor, integument, or fat.

Body Composition Analysis. Prior to tumor implantation, all animals underwent body composition analysis using a Lunar PIXImus densitometer (software version 1.42.006.010; Lunar Corp, Madison, WI). The densitometer uses dual-energy X-ray absorptiometry to provide accurate and precise measurements of fat mass (FM), bone-free lean body mass (LBM), and total bone mineral density (BMD).[16] The analysis was repeated on day 20 in live animals, prior to sacrifice. Pre- and post-tumor results were compared.

Serum Cytokine/Chemokine Assays. On day 2 and at the completion of the experiment, approximately 200 μL of blood was collected from each mouse. Serum was collected after the clotting of blood at room temperature for 1 hour. Measurement of cytokines and chemokines was made using the Bioplex Protein Array system (BioRad Hercules, CA). This is a novel multiplexed, particle-based, flow cytometric assay that utilizes anti-cytokine monoclonal antibodies linked to microspheres incorporating distinct proportions of 2 fluorescent dyes. Our assay was customized to detect and quantify 18 cytokines and chemokines, including IL-1β, IL-1α, IL-6, TNF-α, and IFN-γ, among others. For each cytokine, 8 standards ranged from 2 to 32,000 pg/mL and the minimum detectable dose was <10 pg/mL. Pre- and post-implantation circulating cytokine levels were then compared.

Western Blot Analysis of Tumor-Derived Cachectic Factor, Lipid Mobilizing Factor. Proteins were isolated from the murine adenocarcinoma cells (MAC) 13 and 16 cell lines (kindly provided by Dr. Michael Tisdale), an established animal model of cachexia, and the murine head and neck carcinoma cell line, SCCF, and analyzed for the presence of LMF using western blot analysis. The negative control was the murine adenocarcinoma cell line, MAC13, which does not overexpress LMF or induce cachexia. Samples containing 40 μg of protein were mixed with equal volumes of 2× sodium dodecyl sulfate (SDS) loading buffer, and incubated at 90°C for 5 minutes. Proteins were separated by electrophoresis on 12% SDS-polyacrylamide gels and were then transferred to nitrocellulose membranes. Gels were stained with Ponceau S to confirm equal loading. Immunological detection was performed by using ZAG E-20, a mouse polyclonal antibody (pAb) to mouse ZAG (Santa Cruz Biotechnology) at a 1:250 dilution. Blots were then incubated with a donkey anti-goat secondary Ab conjugated to horseradish peroxidase, at a dilution of 1:1000 (Santa-Cruz Biotechnology). Detection was by enhanced chemiluminescence (Amersham Pharmacia). The size of the proteins detected was estimated using Full Range Rainbow molecular weight markers (Amersham Pharmacia).

Ubiquitin Ligase Expression in Muscles. On postimplantation day 23, mice were euthanized, and hind leg quadriceps and gastrocnemius muscles were rapidly dissected and snap-frozen in liquid nitrogen. Gastrocnemius and quadriceps muscles from saline- and SCCF-injected C3H/HeN mice were homogenized. To confirm the presence of muscle atrophy, isolated muscle tissue was analyzed for the E3 ubiquitin ligase MuRF1 using northern blot analysis, as previously described.[17] Expression of MuRF1 was quantified by phosphoimaging and compared with the expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), a housekeeping gene.

Statistical Analysis. All quantitative data are represented as mean ± SEM. To compare pre- and post-tumor chemokine/cytokine levels, statistical means were calculated using the original scale and the log scale (multiplicative effect log post − log pre = log post/pre). Student's t test was used to compare statistical means. We also calculated 95% confidence intervals. Statistical significance was set at $p < .05$.

RESULTS

Effect of Tumor on Body Weight. Mice were fed ad lib and inoculated with either the SCCF cell line
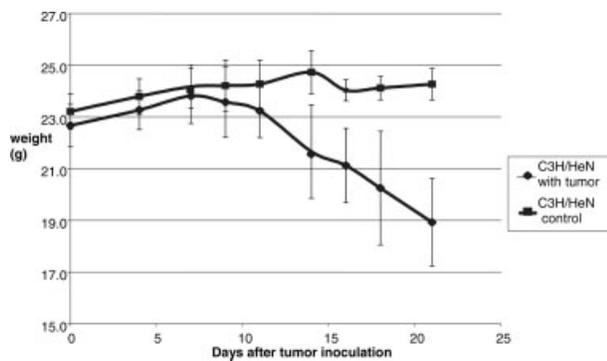
FIGURE 1. Weight loss in mice with tumors. Following injections with either phosphate-buffered saline (control) or SCCF cells, mice were monitored for 22 days. Each group had 5 animals and the experiment was performed in duplicate. The data is expressed as the mean with the SD ($p < .01$).

or saline. They were monitored for changes in weight for 22 days, at which time the control animals had large tumors and signs of severe cachexia. There was a significant weight loss in the animals with tumors when compared with controls. The SCCF-injected mice weighed 18.9 g versus the control group which weighed 26.7 g ($p < .001$) (Figure 1).

Effects of Tumor on Food Intake and Body Weight. In a separate experiment, mice were individually housed. Food intake did not significantly differ in either the SCCF-injected or saline-injected groups ($p = .84$) (Figure 2). Both groups of mice gained weight at the same rate until 12 days after the injection of tumor cells, at which point tumor-bearing mice had an increased percentage of weight gain compared with the sham-injected mice due to the tumor burden. This continued until day 18, when a
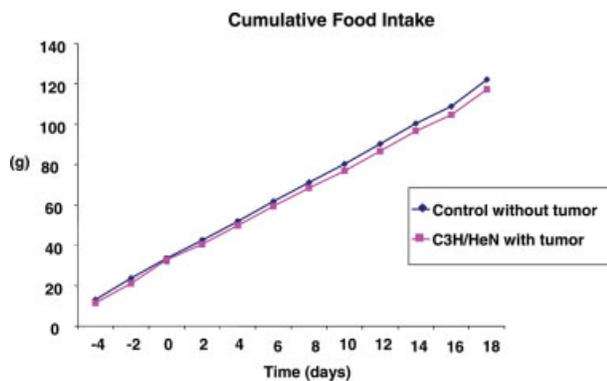

FIGURE 2. The amount of ingested food was measured by taking daily weight of food in grams for each mouse. Daily food intake and cumulative food intake is equal in both groups of mice ($p = .84$). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

sharp decline in percentage of weight gain occurred in the tumor-bearing group. The sham-injected mice reached a plateau in their weight from days 14 to 20. At the end point, the net weight change percent of tumor-bearing mice was lower than that of control mice (Table 1).

The hind legs of the mice were compared to assess muscle mass. The hind legs of the mice were weighed immediately after the mice were euthanized to determine differences in the mass of quadriceps and gastrocnemius muscles. There was a statistically significant difference in the mass of hind legs, with tumor-bearing mice maintaining an average of 0.915 ± 0.09 g and sham-injected mice having an average of 1.068 ± 0.08 g ($p = .02$) (Figure 3).

Body Composition. While FM increased 73% in the saline-injected mice, it increased 53% in tumor-injected mice over the same time period. BMD decreased in tumor-injected mice but increased nearly 9% in sham-injected mice. Interestingly, lean body mass increased in tumor-injected mice when compared with sham-injected mice ($11.0\% \pm 9.3\%$ vs $1.5\% \pm 0.9\%$, respectively) (Table 1). Since the mean tumor mass in tumor-injected mice was 1.83 g and made up approximately 7% of their final body weight, the increase in lean body mass may actually reflect the tumor growth since these are dense tumors.

Table 1. Body composition analysis.

	C3H/HeN with tumor	C3H/HeN control (no tumor)	p value
Final wt, g	24.8 ± 0.4	25.3 ± 0.9	NS
Final average tumor wt, g	1.8 ± 0.9	0	
Tumor as % of final wt	7	0	
Final body wt – tumor, g	23.0	25.3	NS
Net body wt change, %	4.0	14.1	.06
Mean food intake, g	7.8 ± 0.5	8.0 ± 0.7	NS
Last daily food intake, g	8.5 ± 0.4	8.1 ± 0.3	NS
% change final wt	12.3 ± 2.3	13.9 ± 3.6	NS
% change in FM	53.5 ± 21.8	73.36 ± 12.8	NS
% change in BMD	−1.90 ± 1.9	8.6 ± 1.1	0.001
% change in LBM	11.0 ± 9.3	1.5 ± 0.9	NS
Signs of cachexia	+	−	

Abbreviations: FM, fat mass; BMD, bone mineral density; LBM, lean body mass.
Note: Weights are expressed as averages with standard errors. Comparisons between mean changes in FM, BMD made with chi-square analysis. Student's t test used to compare means. Signs of cachexia were defined as wasting of skeleton and limb muscles, lethargy.
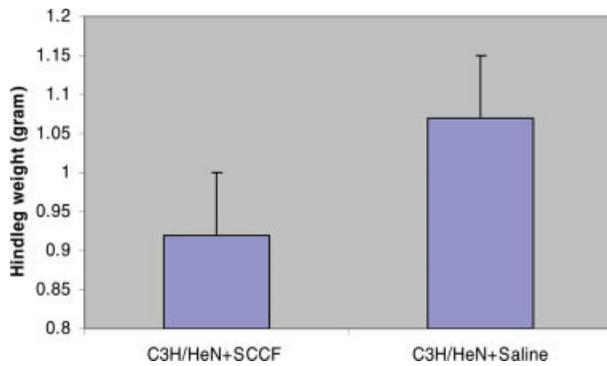
FIGURE 3. The mouse hind legs were weighed immediately after euthanasia. The weight did not include tumor, integument, or fat. Muscle mass decreases in hind legs of mice with tumors ($p = .02$). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Association of Cytokine Levels with Wasting. Measurement of pre- and post-tumor levels of chemokines and cytokines demonstrated that only IL-1β was significantly elevated in SCCF-injected mice at the end of the experiment (73%, $p = .04$). There were no other statistically significant elevated levels of chemokines or cytokines, including IL-6, TNF-α, or IFN-γ ($p = .36, .24, .93$, respectively) (Table 2). IL-1α was significantly decreased in the tumor-bearing mice ($-42\%, p = .02$).

Table 2. Circulating chemokine and cytokine levels in mice, before tumor inoculation and after tumor growth for 23 days.

Cytokines/ chemokines levels	C3H/HeN		
	Pre-tumor	Post-tumor	p value
IL-1β	362.87 ± 25.69	626.68 ± 61.60	.04
IL-1α	234.41 ± 33.69	134.59 ± 9.62	.02
IL-6	144.07 ± 27.35	142.38 ± 6.30	.36
IFN-γ	82.83 ± 16.14	71.17 ± 4.42	.93
TNF-α	3245.53 ± 512.76	2529.31 ± 258.41	.24
IL-2	38.09 ± 1.60	40.81 ± 1.76	.39
IL-3	14.25 ± 1.67	10.70 ± 0.92	.375
IL-4	2.86 ± 0.33	2.80 ± 0.51	.98
IL-5	9.28 ± 1.74	4.65 ± 0.86	.10
IL-10	207.17 ± 37.24	251.09 ± 68.55	.34
IL-12 (p40)	1333.23 ± 179.48	1271.11 ± 55.07	.69
IL-12 (p70)	627.85 ± 112.63	630.61 ± 73.52	.34
IL-17	671.25 ± 144.52	778.84 ± 220.25	.52
GM-CSF	42.74 ± 6.64	42.28 ± 10.56	.66
G-CSF	305.84 ± 75.50	237.24 ± 104.89	.92
KC	212.64 ± 38.89	288.32 ± 66.22	.26
MIP-1α	119.60 ± 11.44	129.72 ± 7.56	.22
RANTES	520.55 ± 41.73	672.72 ± 313.84	.08

Abbreviations: IL, interleukin; IFN-γ, interferon gamma; TNF-α, tumor necrosis factor alpha; GM-CSF, granulocyte macrophage colony stimulating factor; G-CSF, granulocyte colony stimulating factor; KC, mouse chemokine N51; MIP-1α, macrophage inflammatory protein-1 α; RANTES, regulated on activation, normal T expressed and secreted.
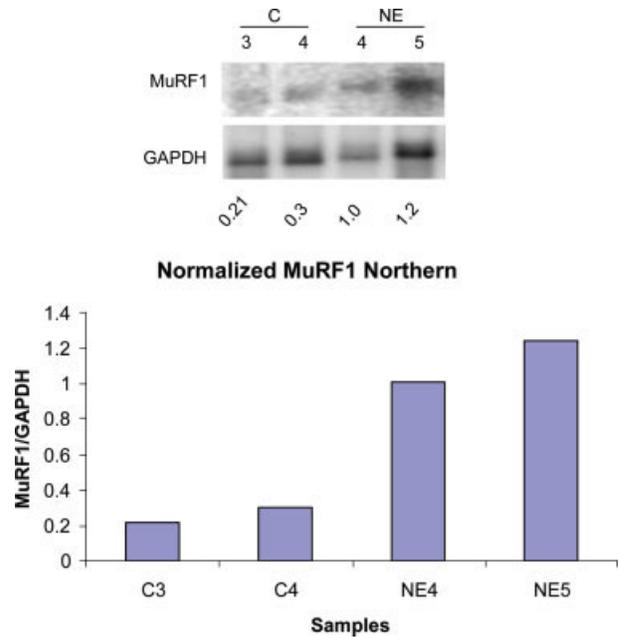

Normalized MuRF1 Northern

FIGURE 4. Marker of muscle atrophy seen in mice with tumors and not in control mice; normalized MuRF1 Northern blot. Abbreviations: C, control mice; NE, C3H/HeN mice with tumor; MuRF1, muscle ring finger 1 ligase; GADPH, glyceraldehyde-3-phosphate dehydrogenase. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Tumor-Induced Ubiquitin Ligase Expression. The E3 ubiquitin ligase MuRF1 targets proteins for degradation in the ubiquitin-proteasome system and is elevated in muscles undergoing protein degradation. When hind leg quadriceps muscles were analyzed for MuRF1, muscles from tumor-bearing mice had elevated levels when compared with muscles from control mice (Figure 4). The same regulation was observed with gastrocnemius muscles (data not shown).

Presence of Lipid Mobilizing Factor in SCCF Tumor Cells. The SCCF cell line was found to express the tumor-derived mediator of cachexia, LMF, a 43-kDa glycoprotein. Western blot analysis demonstrated the presence of LMF in the SCCF cell line and in the positive control, MAC16 cell line, but not in the negative control, MAC13 (Figure 5).
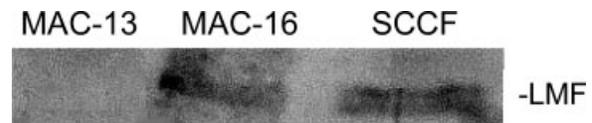

FIGURE 5. Lipid mobilizing factor (LMF), a 43-kDa protein, was detected in SCCF cell line using Western blot analysis. MAC 16 cell line was a positive control and the MAC 13 cell line was used as a negative control. Abbreviations: LMF, lipid mobilizing factor; MAC, murine adenocarcinoma.

DISCUSSION

In this study, we showed that the immunocompetent C3H/HeN mice develop classic signs of cancer cachexia when injected with the syngeneic cell line SCCF. Tumor-injected mice experienced weight loss in the days prior to sacrifice at the point of maximal tumor burden. This weight loss was not related to decreased food intake since the amount of food ingested by the SCCF-injected mice was the same as the control mice. Furthermore, the average daily food intake in the final week was higher in tumor-bearing mice. Comparison of hind leg bone and muscle demonstrated that tumor-bearing mice had significantly decreased bone and muscle mass when compared with control mice. Of the 2 muscle fiber types, it is generally the type II fast-twitch fibers that are frequently atrophied in both humans and animals with cachexia.[17] These muscles are located on the hind leg and include the quadriceps and gastrocnemius muscles. This correlates with the pattern of muscle atrophy seen in humans.

To provide a molecular marker of muscle wasting, the prominent muscles of the hind leg, the quadriceps and gastrocnemius muscles, were analyzed for protein degradation using Northern blots for the E3 ligase, MuRF1. This ubiquitin ligase gene is selectively induced in muscles undergoing atrophy or degradation. Levels of MuRF1 were significantly higher in SCCF-injected animals when compared with controls. This provides compelling evidence for cancer-related muscle wasting in SCCF-injected mice, especially since the mice were ingesting the same amount of food as the controls.

The SCCF cell line in this study was found to secrete LMF, a 43-kDa tumor-derived factor that stimulates lipolysis in adipose tissue and produces cachexia in mice implanted with LMF-producing tumors. This protein, also called ZAG, is upregulated and overexpressed in white adipose tissue in mice with cachexia.[8] Its presence in SCCF tumor cells was related to a decline in FM in tumor-bearing mice. Clinically, tumor-bearing mice appeared much leaner than control mice and this appearance was consistent with muscle wasting. In addition to reduced FM, body composition analysis revealed decreased BMD in the tumor-bearing mice.

In the current animal model, circulating serum cytokine levels were measured in the animals just prior to sacrifice. Only IL-1β was significantly elevated in the tumor-bearing mice when com-pared with controls. Other animal models of cachexia, including the cachexia-inducing C26 model or Lewis lung carcinoma cell lines, implicate contributions from IL-6 and TNF-α, respectively. This is not seen in all models since the cachexia-forming MAC16 tumor cells does not appear to cause cachexia by TNF-α or by actions from IL-6 since antibodies to TNF-α did not ameliorate the cachexia and IL-6 does not appear to be upregulated.[18] Interestingly, in our study, IL-6, TNF-α, and IFN-γ levels were not elevated in cachectic mice. In other peripheral tumor models, proinflammatory cytokine activation was not detectable until 28 days[19] and 36 days[20] after tumor implantation. This could mean that IL-6, TNF-α, and IFN-γ are not mediators of cachexia in this model of head and neck cancer, or that insufficient time has elapsed to develop a chronic inflammatory state. Indeed, it has been suggested that the failure of cytokine activation during cachexia may represent an attempt by the host to contain the inflammatory response and minimize systemic toxicity.[21] Alternatively, this model may represent a model of cancer cachexia in which IL-1β may contribute to the wasting seen. Future studies may use antibodies to IL-1β to determine whether this treatment may be protective of the muscle wasting.

The advantages of establishing an immunocompetent model of head and neck cancer cachexia are numerous. An immunocompetent model allows us to study the contributions of both innate and acquired arms of the immune system. Therefore, we can use this model to determine the contribution of proinflammatory cytokines to the development of cachexia. This immunocompetent model will also allow investigators to obtain pre- and post-tumor (or tumor treatment) body composition analysis since athymic mice cannot be returned to animal labs once they are studied. This model can also be used for screening compounds to combat cancer cachexia. Finally, this model also provides a cost-effective alternative to the use of tumor xenografts in athymic mice for studying cancer cachexia. Thus, this model may be a physiologically relevant and practical model for the elucidation of the etiology of cancer cachexia.

There are limitations to this study. LBM increased in tumor-bearing mice, and since the average tumor weight accounted for approximately 7% of total body weight in these mice, this increase in LBM may reflect the large tumor masses. Evidence that LBM is due in part to

tumor size includes the decreased weight of hind leg muscles and the molecular evidence of muscle atrophy, as described earlier. Analysis of body composition is an important research tool when studying cachexia, but the contribution of the tumor mass in animal models is more difficult to discern. Future experiments should include protocols that resect tumor masses prior to body composition analysis. In addition, the finding of decreased BMD in the mice should be further investigated with experiments that measure the separate weights of muscles and bones of the hind legs, in addition to more dedicated studies of the pathophysiology of bone metabolism during cachexia.

In summary, we demonstrate the novel development of a murine model for head and neck cancer cachexia. Muscle wasting in this model was confirmed by muscle weights and molecular markers of muscle atrophy. Cachexia in this model may be mediated by IL-1β, which was elevated in tumor-bearing mice with evidence of wasting. A tumor-derived catabolic factor, LMF, was produced by the SCCF cell line, and this correlated with reduced fat mass seen on body composition analysis.

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