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Myocardial dysfunction in an animal model of cancer cachexia

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Abstract

Aims—Fatigue is a common occurrence in cancer patients regardless of tumor type or anti-tumor therapies and is an especially problematic symptom in persons with incurable tumor disease. In rodents, tumor-induced fatigue is associated with a progressive loss of skeletal muscle mass and increased expression of biomarkers of muscle protein degradation. The purpose of the present study was to determine if muscle wasting and expression of biomarkers of muscle protein degradation occur in the hearts of tumor-bearing mice, and if these effects of tumor growth are associated with changes in systolic dysfunction.

Main Methods—The colon 26 adenocarcinoma cell line was implanted into female CD2F1 mice and skeletal muscle wasting, *in vivo* heart function, *in vitro* cardiomyocyte function, and biomarkers of muscle protein degradation were determined.

Key Findings—Expression of biomarkers of protein degradation were increased in both the gastrocnemius and heart muscle of tumor-bearing mice and caused systolic dysfunction *in vivo*. Cardiomyocyte function was significantly depressed during both cellular contraction and relaxation.

Significance—These results suggest that heart muscle is directly affected by tumor growth, with myocardial function more severely compromised at the cellular level than what is observed using echocardiography.

Keywords

cancer; cachexia; myocardial function; cardiomyocyte; autophagy; ubiquitin

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Introduction

Cancer cachexia is a syndrome of anorexia, weight loss, and skeletal muscle wasting that occurs in patients during end-stage cancer. One of the paradoxes of this complicated syndrome is that reduced food intake alone does not explain the extent of weight loss or the specific loss of lean body mass. The loss of skeletal muscle mass is thought to contribute to the symptoms of weakness and fatigue (Weber et al. 2009), which in turn negatively affect the functional status and quality of life of patients with cancer cachexia (Fouladi et al. 2007; Wang 2008).

A growing body of evidence from animal models of cancer cachexia has shown that proinflammatory cytokines, such as tumor necrosis factor- α (TNF- α) and interleukin-6 (IL-6), play pivotal roles (Argiles et al. 2009; Saini et al. 2009; Tisdale 2009) in tumor-induced muscle wasting. These cytokines increase expression and activity of the ubiquitin-proteasome (UP) pathway of myofiber degradation (Cohen et al. 2009) and autophagy in skeletal muscle (Mammucari et al. 2007). The UP pathway is characterized by increased expression of MAFbx, a rate-limiting ubiquitin ligase that attaches ubiquitin molecules to the protein to be degraded within the enzyme-rich proteasome (Acharyya and Guttridge 2007). Autophagy is characterized by increased expression of Bnip3, a protein required for the formation of autophagic vesicles for degradation of proteins by cathepsins (Levine and Kroemer 2008). It has been suggested that proteases such as cathepsins are needed for the dismantling of actin-myosin complexes, after which the UP pathway dominates for the further degradation of myosin (Saini et al. 2009). These two major pathways of muscle protein breakdown are independently regulated, though both involve nuclear transcription factors regulating expression of MAFbx and Bnip3 in skeletal muscle (Mammucari et al. 2007; Zhao et al. 2008).

To date, little work has been done to determine whether tumor growth increases expression of MAFbx or Bnip3 in heart muscle. Myocardial dysfunction could play a major role in the symptoms of fatigue and weakness experienced by patients with cancer cachexia (Schunemann et al. 2008). While there is clear evidence that cytotoxic chemotherapy agents can impair myocardial function (Wold et al. 2005), it is not known if tumor growth alone is sufficient to alter myocardial function. The purpose of the present study was to compare expression of MAFbx and Bnip3 in gastrocnemius and heart muscle of mice bearing the colon26 adenocarcinoma, a widely used animal model of cancer cachexia. We used techniques of echocardiography to examine heart function *in vivo*, followed by *in vitro* studies of contractile function of individual cardiomyocytes isolated from tumor-bearing and healthy control animals.

Material and Methods

Animal Model

Twenty-four pathogen-free CD2F1 adult female mice weighing 18–20 grams were obtained from Harlan (Indianapolis, IN). The mice were maintained on a 12 hour light-dark cycle that commenced at 6 a.m. and were housed three to a cage to reduce isolation stress. Animals were acclimated to their housing for three days before the start of the experiment which was approved by the Institutional Animal Care and Use Committee of the Ohio State University. The colon26 adenocarcinoma (colon26) cell line was maintained in culture as previously described (Graves et al. 2006). Cells were harvested using 0.05% trypsin-EDTA (Gibco), washed in phosphate buffered saline (PBS), counted using 0.04% trypan blue, and resuspended at 2.5×10^6 cells/ml in PBS. Mice were gently restrained and injected subcutaneously between the scapulae with 0.2ml of cell suspension (n=12) or PBS (n=12). Growth of the tumor was detectable by day 7, and mice become moribund by day 21. Tumor

growth on the upper back did not impair the animal's mobility or access to food and water. In this animal model of cancer cachexia, voluntary wheel running activity of the tumor-bearing mice progressively declines, a measure of fatigue in mice (Wood et al. 2006) (Skinner et al. 2009) after day 14, and food intake and body weight do not decline until after day 20 (Graves et al. 2005; Hitt et al. 2005). In the present study, body weight and food and water intake were monitored once a week for two weeks, and every other day from day 14 to 19 of tumor growth.

On day 19, the animals were euthanized using inhaled CO₂ gas followed by cervical dislocation as specified by the American Veterinary Medicine Association Panel on Euthanasia. Each animal was weighed, and heart and gastrocnemius muscles were carefully dissected, weighed, and frozen in liquid nitrogen. Lastly, the tumor was removed and weighed. The weights of the right and left gastrocnemius muscles were averaged and the gastrocnemius and heart weights were normalized to the intact body weight since the tumor mass contributes to the load on both the gastrocnemius and heart muscle.

Gene expression

Total RNA was extracted from 100 mg of frozen gastrocnemius or heart muscle in 1 ml of TRIzol (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions and as previously described (Graves et al. 2006). 1 µg RNA was treated with DNase 1 (Invitrogen) and reverse transcribed to cDNA using the Iscript cDNA synthesis kit (BioRad). Real time PCR was performed using primer pairs for MAFbx (Forward 5'-GTGCTTACAACCTGAACATCATGC A3'; Reverse 5'-TGGCCCAGGCTGACCA3'), GAPDH (Forward 5'-ATG GTG AAG GTC GGT GTG AAC GG-3'; Reverse 5'-AGG GGT CGT TGA TGG CAA CAA TCT-3'), and SYBR super mix (BioRad). Bnip3 was detected using a TaqMan[®] Gene Expression Assay (Hs00969291; ABI Product Number: Mm01275601_g1) according to the manufacturer's instructions. All reactions were performed in duplicate using 25 ng of cDNA in a final reaction volume of 25 µl using the iCycler iQ5 (BioRad). The reaction conditions were 95°C for 15s and 60°C for 1 min for 40 cycles after the initial denature at 95°C for 10 min. The results for MAFbx and Bnip were normalized to GAPDH and expressed as $2^{-\Delta\Delta CT}$ (normalized expression ratio).

Echocardiography

A VisualSonics Vevo 2100 Ultra High Resolution *In Vivo* Imaging System (VisualSonics, Toronto, ON, Canada) was used for assessment of cardiac function in ten tumor-bearing and eight control mice. Mice were anesthetized with 1.5% isoflurane and placed on a warming pad. A 3-lead ECG was used to monitor heart rate (VisualSonics). Scanning was performed at a frequency of 30MHz and three total measures from different cardiac cycles were averaged according to standards set forth by the American Society for Echocardiography. M-mode images were obtained at the level of the papillary muscles to assess chamber structure (LV systolic diameter (LVSD), LV diastolic diameter (LVDD)), and LV posterior wall thicknesses (PWT). Systolic function was assessed using M-mode calculations of fractional shortening ($FS = (LVDD - LVSD) / LVDD$). Pulsed wave Doppler in the four chamber apical view was used to assess mitral valve inflow velocities with the sample being taken at the point of maximal flow, as assessed by color Doppler. The E/A ratio was calculated from the mitral valve inflow velocities in order to assess diastolic function. From the same position, we obtained isovolumetric relaxation (IVRT), isovolumetric contraction (IVCT), and aortic ejection time (ET), which was used to calculate the Tei index ($Tei = (IVRT + IVCT) / ET$), a measure of global cardiac function encompassing systolic and diastolic parameters. Total time for each individual echocardiographic analysis was approximately 20 minutes.

Isolation of ventricular myocytes

Four tumor-bearing and four control animals were weighed and injected with heparin sodium (1000IU/kg, i.p.) followed by sodium pentobarbital (50mg/kg, i.p.) The heart was removed and placed on ice in perfusion buffer (pH=7.4) containing (in mM): 113 NaCl, 4.7 KCl, 0.6 KH₂PO₄, 0.6 Na₂HPO₄, 1.2 MgSO₄·7H₂O, 0.032 phenol red, 12 NaHCO₃, 10 KHCO₃, 10 HEPES, and 30 Taurine. The heart was retrogradely perfused through the aorta with buffer containing 0.25mg/ml liberase DH (Roche), 0.14mg/ml Trypsin 2.5% (Gibco) and 12.5μM calcium chloride for five to seven minutes. LV tissue was dissociated by repeated pipetting in 10ml of stopping buffer solution [Perfusion buffer + 10% FBS (Hyclone) + 12.5μM CaCl₂]. The free cells were filtered through a sterile, 100 μm nylon mesh filter and treated with increasing concentrations of CaCl₂ solution (150 μl of 10 mM CaCl₂, 300 μL of 10 mM CaCl₂, 90 μL of 100 mM CaCl₂, and 150 μl of 100 mM CaCl₂) at four minute intervals. The cells were centrifuged and the pellet was re-suspended in 10ml of plating media (Invitrogen, 0.9× MEM; FBS, 5%; 2,3 butanedione monoxime (BDM), 10mM; penicillin & streptomycin, 100U/ml; and L-glutamine, 2mM). An aliquot of cells was placed in a glass-bottom chamber insert (Cell Micro Controls) pre-coated with laminin (Invitrogen) and incubated at 37°C with 5% CO₂ for one hour. Following incubation, culture media (Invitrogen, 1× MEM; BSA 0.1mg/ml; penicillin & streptomycin 100U/ml; L-glutamine 2mM) was added to the chamber.

Measurement of cardiomyocyte function

The culture dishes were loaded onto the stage of an inverted microscope (Olympus IX-70, Olympus Optical Corporation, Tokyo, Japan). The cells were perfused with heated contractile buffer ((131mM NaCl, 4mM KCl, 10mM HEPES, 1mM CaCl₂, 1mM MgCl₂, and 10mM Glucose) at 37°C, and stimulated with a suprathreshold voltage using two platinum wires at a frequency of 1.0Hz, 3msec attached to a FHC stimulator (FHC Incorporation, Bowdoinham, ME, USA). Data were acquired using the Soft Edge MyoCam[®] system (IonOptix Corporation, Milton, MA, USA) using an IonOptix Myocam camera with SoftEdge sarcomere software (IonOptix Corp., Milton, MA, USA). Cell shortening (a measure of cellular systolic function) and re-lengthening (a measure of cellular diastolic function) were assessed using the following indices: peak shortening normalized to baseline sarcomere length (%PS), time-to-90% shortening (TPS-90), time-to-90% relengthening (TR-90), and the maximal velocities of shortening and relengthening (±dL/dt).

Data Analyses

All data are reported as mean ± SEM, and were analyzed using a Student's t-test for independent samples, with p<0.05 considered statistically significant.

Results

Muscle Weights

As shown in Table 1, both absolute and normalized gastrocnemius weight were significantly reduced in tumor-bearing mice compared to healthy controls. However, absolute and normalized heart weights were not different between groups.

Gene expression assessed by RT-PCR

Expression of MAFbx, but not Bnip3, was significantly increased in the gastrocnemius muscle (Figure 1A) of tumor-bearing mice compared to control mice (7.98 ± 2.0 vs 1.23 ± .38, p<0.01). As shown in Figure 1B, expression of MAFbx and Bnip3 mRNA significantly increased in heart muscle of tumor-bearing mice compared to controls.

Echocardiography

No difference was found in LVDD (left ventricular diastolic diameter), however LVSD (left ventricular systolic diameter) was increased significantly in tumor-bearing mice (2.716 ± 0.4111 mm vs 2.078 ± 0.1815 mm in controls, $p < 0.05$; Figure 2D). As shown in Figure 3A, contractility was severely impaired as indicated by a decrease in %FS in tumor-bearing mice (28.44 ± 4.180 vs. 41.18 ± 5.008 in controls, $p < 0.01$). Furthermore, diastolic posterior wall thickness (PWT) was decreased significantly in tumor-bearing mice (0.5997 ± 0.090 mm vs. 0.7575 ± 0.1147 mm in controls, $p < 0.05$; Figure 3B). No significant differences in heart rate, mitral valve E/A ratio, or the Tei index were observed (Table 1).

Cardiomyocyte function

As shown in Figure 4, time to 90% sarcomere contraction (TPS90; Figure 4B) was significantly increased in cardiomyocytes isolated from tumor-bearing mice compared to control mice, and time to 90% relengthening (TR90; Figure 4C) was also increased. These changes were evident without a change in peak shortening normalized to baseline sarcomere length (Figure 4A).

Discussion

Skeletal muscle wasting plays a major role in the morbidity and mortality of cancer cachexia. In the present study, we used the colon26 animal model of cancer cachexia to determine if tumor-induced muscle wasting also occurs in the heart muscle. We observed reduced muscle weight and increased expression of MAFbx in the gastrocnemius muscle of tumor-bearing mice as previously reported by us and others (Gorselink et al. 2006; Graves et al. 2006; Hitt et al. 2005; Tian et al. 2010; van Norren et al. 2009). While heart weight was not reduced in the tumor-bearing mice, expression of MAFbx and Bnip3 mRNA were significantly increased compared to heart tissue from control mice. Echocardiography revealed that LVSD was significantly increased and percent fractional shortening was significantly reduced in tumor-bearing mice, suggesting early systolic dysfunction of the heart.

Our echocardiographic evidence of systolic dysfunction was corroborated by *in vitro* studies of cardiomyocytes isolated from the left ventricle of tumor-bearing and control mice. We observed an increase in time to 90% peak shortening (TPS90) as well as an increase in time to 90% baseline relaxation (TR90). The latter is indicative of diastolic dysfunction, which was not yet evident by *in vivo* echocardiography. We conclude that tumor growth directly alters myocardial function at the cellular level, and alterations in cardiomyocyte function underlie changes in myocardial function detectable *in vivo* by echocardiography.

Others have observed reduced heart weight and systolic dysfunction in severely cachectic tumor-bearing rats (Marin-Corral et al. 2010; Springer 2008) and mice (Tian et al. 2010). In the present study, we found that gastrocnemius muscle weight was reduced and expression of MAFbx, a biomarker of muscle protein degradation, was increased in tumor-bearing mice that were not severely cachectic, e.g. no decline in food intake or body weight. Given the increased expression of MAFbx and Bnip3, another biomarker of muscle protein degradation, in hearts of tumor-bearing mice in the present study, it is likely that wasting of cardiac muscle may have developed if the mice had been allowed to become severely cachectic.

Echocardiography has been used previously to demonstrate myocardial dysfunction in tumor bearing rats (Springer 2008) and mice (Tian et al. 2010). In the present study, we observed an increase in LVSD and decrease in %FS, indicative of systolic dysfunction, with no *in vivo* evidence of diastolic dysfunction. Posterior wall thickness (PWT) was significantly

decreased in tumor-bearing mice, suggesting possible regional wall motion abnormalities and/or remodeling of the ventricle. Tian et al. (Tian et al. 2010) also observed reduced %FS in tumor-bearing mice, however measures of diastolic function were not reported. Our echocardiography data of systolic dysfunction were corroborated by *in vitro* studies of contractile function of cardiomyocytes isolated from the left ventricle of tumor-bearing and control mice. Interestingly, the functional performance of isolated cardiomyocytes suggested an unmasking of diastolic dysfunction that was not detectable by *in vivo* echocardiography.

Several explanations for the decline in contractile kinetics of cardiomyocytes can be derived from the literature. Tian et al. (Tian et al. 2010) observed reduced expression of troponin I mRNA and increased levels of the fetal isoform of myosin heavy chain (MHC- β). Marin-Corral et al. (Marin-Corralet et al. 2010) observed increased oxidation of proteins involved in glycolysis, ATP production, muscle contraction, and mitochondrial function in the heart and hind limb muscles of tumor-bearing rats. To our knowledge, this is the first study demonstrating that tumor growth directly impacts contractile function in cardiomyocytes. Taken together, these data suggest that cardiac evaluation should be a component of the clinical evaluation of cancer patients (Schunemann et al. 2008).

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Figure 1. Expression of MAFbx, and Bnip3 in gastrocnemius (A) and heart (B) muscles of control and tumor-bearing mice normalized to GAPDH expression. *p<0.05.

Figure 2.
In vivo echocardiographic assessment of control (n=8) and tumor-bearing (n=10) mice on day 19 following colon26 adenocarcinoma implantation. Figure 2A–B. Representative M-mode echocardiographic images taken at the mid-papillary level. Figure 2C. Left ventricular diastolic diameter (LVDD) was not different between groups. Figure 2D. Left ventricular systolic diameter (LVSD) was significantly increased in tumor-bearing mice compared to control.

Figure 3. Echocardiographic calculations of control (n=8) and tumor-bearing (n=10) mice on day 19 following colon26 adenocarcinoma implantation. Figure 3A. Percent fractional shortening (%FS) was significantly decreased in tumor-bearing mice compared to control mice, indicating systolic dysfunction *in vivo*. Figure 3B. Posterior wall thickness (PWT) was also significantly decreased in tumor-bearing mice compared to control mice. *p<0.05.

Figure 4.

In vitro cardiomyocyte function in control and tumor-bearing mice. Figure 4A. Peak shortening (PS) normalized to baseline sarcomere length was not different in myocytes from control and tumor-bearing mice. Figure 4B. Time-to-90% shortening (TPS90) was significantly increased in myocytes from tumor-bearing mice, indicating systolic dysfunction at the cellular level. Figure 4C. Time-to-90% relengthening (TR90) was significantly increased in myocytes from tumor-bearing mice, indicating significant diastolic dysfunction at the cellular level. n=36–44 cells from four mice/group, *p<0.05.

Table 1

Body weight and absolute and normalized weights of the gastrocnemius and heart muscles of tumor-bearing (n=12) and control mice (n=12).

	Body weight at sacrifice (g)	Gastrocnemius weight (mg)	Normalized Gastrocnemius weight (mg/g)	Heart weight (mg)	Normalized Heart Weight (mg/g)
Tumor (n=12)	20.4 ± 2.0 *	93.88 ± 7.4 *	4.62 ± .4 *	109.33 ± 11.5	5.36 ± .4
Control (n=12)	21.7 ± 1.1	109.15 ± 7.5	5.04 ± .4	112.08 ± 10.0	5.18 ± .5

* p<0.05

Aortic velocity (Ao Vel), Pulmonary velocity (Pul Vel), ratio between early (E) and late (A) atrial filling, Tei index, and systolic posterior wall thickness (PWT sys) in control and tumor-bearing mice.

Table 2

	HR (beats/min)	Ao Vel (mm/s)	Pul Vel (mm/s)	E/A ratio	Tei index	PWT sys (mm)
Control (n=8)	473.3 ± 6.0	688.3 ± 41.6	-631.1 ± 24.8	1.65 ± 0.12	0.55 ± 0.03	1.03 ± 0.04
Tumor (n=10)	462.8 ± 3.0	717.9 ± 28.1	-569.4 ± 40.1	1.57 ± 0.10	0.65 ± 0.07	0.85 ± 0.05*

* p<0.05