2-28-2011

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Accepted version. *Life Sciences*, Vol. 88, No. 9-10 (February 28, 2011): 406-410. DOI.
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Myocardial dysfunction in an animal model of cancer cachexia

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Running head: Cancer cachexia and the heart

Abstract

Aims: Fatigue is a common occurrence in cancer patients regardless of tumor type or anti-tumor therapies, and may persist for years following the completion of treatment. In rodents, tumor-induced fatigue is associated with a progressive loss of muscle mass, increased expression of biomarkers of muscle protein degradation, and alterations in myosin isoforms that reduce muscle resistance to fatigue. The purpose of the present study was to determine if muscle wasting and muscle protein degradation occurs in the hearts of tumor-bearing mice. **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**: Implantation of the adenocarcinoma cell line at day 19 increased biomarkers of protein degradation in the gastrocnemius and heart muscle and caused systolic dysfunction *in vivo*, with no change in heart weight or mass. Cardiomyocyte function was significantly depressed during both cellular contraction and relaxation. **Significance**: These results suggest that skeletal and heart muscle are both directly affected by tumor growth, with myocardial function more severely compromised at the cellular level than what is observed by echocardiography.

Key words: cancer, cachexia, myocardial function, cardiomyocyte, autophagy, ubiquitin

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 (Fouladiun et al. 2007; Wang 2008).

While there are several hypothesized explanations for muscle wasting in cancer patients, a growing body of evidence from animal models of cancer cachexia suggests that proinflammatory cytokines, such as tumor necrosis factor-alpha (TNF-α) and interleukin-6 (IL-6), play pivotal roles (Argiles et al. 2009; Saini et al. 2009; Tisdale 2009). These cytokines increase expression and activity of the ubiquitin-proteasome (UP) pathway of myofiber degradation (Cohen et al. 2009) and autophagy (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). Unlike proteasomal degradation, which degrades only ubiquitinated protein molecules, autophagy involves small phagosomes located around the nucleus of the cell and between myofibrils (Attaix and Bechet 2007). 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 (Sainiet al. 2009). These two major pathways of muscle protein breakdown are independently regulated, though both involve cytokine-activation of the FoxO3 (Forkhead box-containing protein, O subfamily) nuclear transcription factor (Mammucariet al. 2007; Zhao et al. 2008). The importance of FoxO3 for autophagy-based regulation of cell size, viability, and metabolism in the heart is just recently emerging (Ferdous et al. 2010).

To date, little work has been done to determine whether tumor growth increases expression of biomarkers of the UP pathway or autophagy in heart muscle. Myocardial dysfunction could play a major role in the symptoms of fatigue and weakness of 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 (gastroc) 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). Tumor cell growth on the upper back did not impair the animal's mobility or access to food and water. Body weight and food and water intake were monitored once a week for two weeks, and every other day from day 14 to 18 of tumor growth.

On day 19 of tumor growth, the animals were euthanized using inhaled CO_2 gas followed by cervical dislocation as specified by the American Veterinary Medicine Association Panel on Euthanasia. Each animal was weighed, and the spleen, heart, and gastroc muscles were carefully dissected, weighed, and frozen in liquid nitrogen. Lastly, the tumor was removed and weighed. The weights of the right and left gastroc muscles were averaged to determine muscle weight. Gastroc and heart weight were normalized to body weight to determine muscle mass.

Gene expression

Total RNA was extracted from 100 mg of frozen gastroc or heart muscle in 1 ml of TRIzol (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions and as previously described (Graveset al. 2006). 1 µg RNA was treated with DNase 1 (Invitrogen) and reverse transcribed to cDNA using the Iscipt cDNA synthesis kit (BioRad). Real time PCR was performed using primer pairs MAFbx (Forward 5'GTGCTTACAACTGAACATCATGC A3'; Reverse 5'TGGCCCAGGCTGACCA3'), IL-6 (Forward 5'GCCAGAGTCCTTCAGAGAGATACAGAAACTC3'; Reverse 5'AGCCACTCCTTCTGTGACTCCAGCTTA3') and SYBR super mix (BioRad). Bnip3 was detected using a TaqMan® Gene Expression Assay (Hs00969291) 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 95ºC for 15s and 60ºC for 1 min for 40 cycles after the initial denature at 95ºC for 10 min. The results were normalized to GAPDH and expressed as 2^ – ΔΔ CT (normalized expression ratio).

Echocardiograpy

A VisualSonics Vivo 2100 Ultra High Resolution In Vivo Imaging System (VisualSolics, Toronto, ON, Canada) was used for assessment of cardiac function in nine tumor-bearing and seven 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 20MHz 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) and 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 KH_2PO_4, 0.6 Na_2HPO_4, 1.2 $MgSO_4$-$7H_2O$, 0.032 phenol red, 12 $NaHCO_3$, 10 $KHCO_3$, 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 $CaCl_2$]. The free cells were filtered through a sterile, 100 µm nylon mesh filter and treated with increasing concentrations of $CaCl_2$

solution (150 µl of 10 mM $CaCl_2$, 300 µL of 10 mM $CaCl_2$, 90 µL of 100 mM $CaCl_2$, and 150 µl of 100 mM $CaCl_2$) at four minute intervals. The cells were centrifuged and the pellet was re-suspended in 10ml of plating media (Invitrogen, 0.9X 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% CO_2 for one hour. Following incubation, culture media (Invitrogen, 1X 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 $CaCl_2$, 1mM $MgCl_2$, and 10mM Glucose) at 37ºC, 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 ± SD, 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, gastroc muscle weight and muscle mass were significantly reduced in tumor-bearing mice compared to healthy controls. However, neither heart muscle weight nor mass were different between groups.

Gene expression assessed by RT-PCR

Expression of MAFbx was significantly increased in gastroc muscle of tumor-bearing mice compared to control mice (7.98 ± 2.0 vs 1.23 ± .38, $p<0.01$); expression of IL-6 and Bnip3 were not significantly different between groups. As shown in Figure 1, expression of MAFbx, IL-6, and Bnip3 mRNA were 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 (2.737 ± 0.4304 vs 2.079 ± 0.1961 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 (28.68 ± 4.227 vs. 41.72 ± 5.145 in controls, $p< 0.01$). No significant group differences were found in LV wall thickness, however a trend was observed for a decrease in posterior wall thickness (PWT; $p=0.05$, Figure 3B) in tumor-bearing mice. No significant differences in heart rate, mitral valve E/A ratio, or the Tei index were observed.

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 mass and increased expression of MAFbx in the gastrocnemius muscle of tumor-bearing mice as previously reported by us and others (Gorselink et al. 2006; Graveset al. 2006; Hitt et al. 2005; Tian et al. 2010; van Norren et al. 2009). While heart muscle mass was not reduced in the tumor-bearing mice, expression of MAFbx, IL-6, and Bnip3 mRNA in the heart muscle was significantly elevated 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), indicative of both systolic and diastolic dysfunction, which was not yet evident by *in vivo* echocardiography. We conclude that tumor growth 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 mass and systolic function in severely cachectic tumor-bearing rats (Marin-Corral et al. 2010; Springer 2008) and mice (Tianet al. 2010) coupled with significant reductions in food intake and body weight compared to controls.

In the latter study, the reduction in systolic function was not seen in healthy controls pair fed to match the food intake of the tumor-bearing mice. In the present study, mice were sacrificed before signs of weight loss and anorexia were evident. Taken together, these data suggest that tumor growth, and not weight loss or anorexia, drives the decline in myocardial function in this animal model of cancer cachexia. Given the increased expression of MAFbx, IL-6, and Bnip3 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 followed for a longer period of time. It is intriguing that the studies showing reduced heart mass, anorexia, and body weight loss in tumor-bearing rats and mice were conducted in male animals, while the present study, which did not find reduced heart mass, was conducted in female tumor-bearing mice. Others have observed more severe weight loss in males than females in an animal model of cardiac cachexia (Palus et al. 2009). More research is needed to determine if gender affects the progression of cardiac or skeletal muscle wasting in animal models of cancer cachexia.

Echocardiography has been used previously to demonstrate myocardial dysfunction in tumor bearing rats (Springer 2008) and mice (Tianet 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) trended towards a significant different among groups, however this may be due to the small sample size in the present study. Tian et al. (Tianet al. 2010) also observed reduced %FS in tumor-bearing mice, but did not report measures indicative of diastolic function. 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. (Tianet al. 2010) observed marked fibrosis and disrupted myocardial untrastructure in hearts of tumor-bearing mice compared to healthy controls. This was accompanied by 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 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 impaired contractile function in cardiomyocytes from tumor-bearing mice. Taken together, these data suggest that cardiac evaluation should be a component of the clinical evaluation of cancer-related fatigure (Schunemannet al. 2008).

Acknowledgements

This study was funded in part by the Oncology Nursing Society Foundation and NIH/NINR R15 NR010801 (D.O.M.).

Figure Legends

Figure 1. Expression of MAFbx, IL-6 and Bnip3 in heart muscle of control and tumor-bearing mice normalized to GAPDH expression. *p<0.05.

Figure 2. *In vivo* echocardiographic assessment of control and tumor-bearing 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 and tumor-bearing 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 not different between groups. *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. *$p<0.05$.

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