Journal of Molecular and Cellular Cardiology 85 (2015) 37–47

Contents lists available at ScienceDirect

Journal of Molecular and Cellular Cardiology

journal homepage: www.elsevier.com/locate/yjmcc

Original article

# Losartan treatment attenuates tumor-induced myocardial dysfunction

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ARTICLE INFO

*Article history:*
Received 9 March 2015
Received in revised form 28 April 2015
Accepted 11 May 2015
Available online 16 May 2015

*Keywords:*
Cancer cachexia
Cardiovascular function
Calcium signaling
Losartan
Cardiomyocyte

ABSTRACT

Fatigue and muscle wasting are common symptoms experienced by cancer patients. Data from animal models demonstrate that angiotensin is involved in tumor-induced muscle wasting, and that tumor growth can independently affect myocardial function, which could contribute to fatigue in cancer patients. In clinical studies, inhibitors of angiotensin converting enzyme (ACE) can prevent the development of chemotherapy-induced cardiovascular dysfunction, suggesting a mechanistic role for the renin–angiotensin–aldosterone system (RAAS). In the present study, we investigated whether an angiotensin (AT) 1-receptor antagonist could prevent the development of tumor-associated myocardial dysfunction. Methods and results: Colon26 adenocarcinoma (c26) cells were implanted into female CD2F1 mice at 8 weeks of age. Simultaneously, mice were administered Losartan (10 mg/kg) daily via their drinking water. In vivo echocardiography, blood pressure, in vitro cardiomyocyte function, cell proliferation assays, and measures of systemic inflammation and myocardial protein degradation were performed 19 days following tumor cell injection. Losartan treatment prevented tumor-induced loss of muscle mass and in vitro c26 cell proliferation, decreased tumor weight, and attenuated myocardial expression of interleukin-6. Furthermore, Losartan treatment mitigated tumor-associated alterations in calcium signaling in cardiomyocytes, which was associated with improved myocyte contraction velocity, systolic function, and blood pressures in the hearts of tumor-bearing mice. Conclusions: These data suggest that Losartan may mitigate tumor-induced myocardial dysfunction and inflammation.



## 1. Introduction

Cancer cachexia, a syndrome consisting of fatigue, muscle wasting, and weight loss with or without anorexia, is observed in a large percentage of cancer patients with incurable disease [1] and contributes to 22% of cancer deaths [1,2]. New research by our lab and others has shown that cancer cachexia involves not only the loss of skeletal muscle, but can also cause pathologic alterations within the heart [2,3]. The resultant effects on myocardial function likely contribute to fatigue and decreased quality of life in these patients.

We and others have demonstrated tumor-induced cardiac remodeling and myocardial dysfunction [2–4]. Tumor-induced cardiac remodeling involves increased expression of pro-inflammatory cytokines, such as interleukin-6 (IL-6) [5], as well as ventricular wall thinning and decreased troponin I levels (a protein involved in cardiac contraction) [2]. Previous work from our lab has also shown increased expression of MAFbx mRNA, a muscle-specific ubiquitin ligase involved in the ubiquitin proteasome pathway (UPP) of protein degradation, and Bnip3, a protein involved in the formation of autophagic vesicles, in mice inoculated with the colon26 (c26) adenocarcinoma cell line [3]. We also recently showed that IL-6 levels are elevated in both the serum and heart muscle in this model [3]. Together, these results indicate an increase in muscle protein degradation and inflammation in the heart, and myocardial dysfunction due to growth of the c26 adenocarcinoma cells.

The current data regarding the impact of the renin–angiotensin–aldosterone system (RAAS) on the development of cancer-induced myocardial dysfunction and its potential therapeutic properties are inconclusive. Angiotensin II receptor subtypes 1 and 2 ($AT_1$ and $_2$) have direct effects on tumor development through the induction of cell proliferation and vascular endothelial growth factor (VEGF)-induced angiogenesis [6]. Furthermore, the $AT_2$ receptor antagonist Losartan has been shown to limit tumor-associated angiogenesis, inhibit collagen synthesis, and attenuate tumor progression. Additionally, Losartan improves

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http://dx.doi.org/10.1016/j.yjmcc.2015.05.007
0022-2828/

the distribution and efficacy of nanotherapeutics in tumor therapy [7]. Recently, a clinical trial revealed that treatment with a high dose of the ACE inhibitor Enalapril and also beta-receptor blocker treatment with carvedilol prevented the development of chemotherapeutic-induced myocardial dysfunction [8]. These studies suggest a mechanistic role for the RAAS in cancer treatment-induced myocardial dysfunction [9]. Experimental studies reported preventative effects of beta-blockers and aldosterone antagonists but not angiotensin-converting-enzyme (ACE) inhibitors on loss of body weight and skeletal muscle mass, as well as tumor-induced alterations in cardiac dimensions in a mouse model of liver cancer-induced cardiac cachexia and muscle wasting [10]. More recent studies indicated that combined treatments of chemotherapeutics with $AT_1$ receptor antagonists improved survival [11]. However, few studies have investigated the role of the RAAS in the direct effects of tumor progression and subsequent myocardial dysfunction and potential therapeutic prospects.

The RAAS is known to play a major role in myocardial remodeling and dysfunction. Recently, Angiotensin II (AngII) has been implicated in skeletal muscle catabolism in tumor-bearing animals via activation of the UPP of myosin protein degradation. These data suggest that the RAAS could play a role in tumor-induced myocardial dysfunction [12–17]. In order to examine the impact of the RAAS in tumor-induced myocardial dysfunction and potential therapeutic feasibility, we treated c26 tumor-bearing mice with Losartan (LOS), an $AT_1$ antagonist, and examined in vivo and in vitro myocardial function, blood pressures, and AngII serum concentration following cancer cachexia development.

## 2. Materials and methods

### 2.1. Animal model

Animal protocols were approved by the Institutional Animal Care and Use Committee (IACUC) at the Research Institute at Nationwide Children's Hospital and The Ohio State University. One hundred adult (8 week old) female CD2F1 mice (Harlan) were handled in accordance with NIH guidelines and housed in a specific pathogen free facility, five per cage on a 12 hour light/dark cycle. Half of the mice were inoculated with c26 tumor cells (tumor) and half injected with a similar saline volume served as healthy controls (control). The c26 cells were cultured and injected subcutaneously above the scapula, as previously described [3]. Half of the tumor (tumor/LOS) and half of the control (control/LOS) mice were administered 10 mg/kg of Losartan (LOS) daily via their drinking water, beginning on the day of tumor cell injection.

Animals were euthanized on day 19 after tumor cell injection via pentobarbital injection (20 mg/kg for myocyte isolation) or carbon dioxide inhalation followed by cervical dislocation for tissue collection as approved by the American Veterinary Medicine Association Panel on Euthanasia. Hearts were removed, weighed and then used for primary cardiomyocyte isolation or snap-frozen in liquid nitrogen for molecular analyses. Gastrocnemius muscles and tumors were removed, weighed, and snap-frozen in liquid nitrogen.

### 2.2. Real-time polymerase chain reaction

Total RNA was extracted from 100 mg cardiac tissue as previously described [3]. Real time PCR (RT-PCR) for MAFbx (forward 5′-GTGC TTACAACTGAACATATGCA-3′; reverse 5′-TGGCCCAGGCTGACCA-3′), GAPDH (forward 5′-ATGGTCAAGGTCGGTGTGAACGG-3′; reverse 5′-AGGGGTGGTTGATGGCAACAATCT-3′) and IL-6 (forward 5′-GCCAGA GTCCTTCAGAGAGATACAGAAACTC-3′; reverse 5′-AGCCACTCCTTCTG TGACTCCAGCTTA-3′) was performed using primer pairs and SYBR super mix (BioRad). The TaqMan Gene Expression Assay was used to detect gene expression of Bnip3 according to the manufacturer's instructions. Briefly, 25 μl samples were run in duplicate in an iCycler iQ5 (BioRad) for 40 cycles at 95 °C for 15 s and 60 °C for 1 min after the initial 10 min 95 °C denaturation period. MAFbx, Bnip3, and IL-6 levels were all normalized to GAPDH expression [3].

### 2.3. Plasma cytokines

Plasma was obtained from mice using an abdominal aortic stick immediately following euthanasia. Blood was collected in EDTA tubes and centrifuged at 1500 ×*g*, 10 min, 4 °C to separate the plasma. Plasma levels of interleukin (IL)-1, IL-6, IL-10, IL-12, interferon-gamma (IFN-γ), tumor necrosis factor-α (TNF-α), and chemokine (C–X–C motif) ligand 1 (CXCL1) were measured using the MSD Mouse Pro-inflammatory 7-Plex Ultra-Sensitive Kit (Meso Scale Discovery K15012C) according to the manufacturer's instructions.

### 2.4. Echocardiography

On day 19 post-injection, in vivo cardiac function was assessed in 56 mice (15 control, 14 control/LOS, 17 tumor, and 10 tumor/LOS) via echocardiography using a VisualSonics Vevo 2100 Ultra High Resolution In Vivo Imaging System (VisualSonics, Toronto, ON, Canada) as previously described [3,18]. Briefly, mice were anesthetized with 1.5% isoflurane in an anesthesia induction chamber. Following the induction of anesthesia, mice were placed on a warming pad, and connected to a three lead electrocardiogram monitor. During echocardiographic analyses, mice were continuously sedated with 1.0% isoflurane (in 100% $O_2$) to receive identical anesthetic conditions for all groups. Left ventricular systolic diameter ($LVES_d$), left ventricular diastolic diameter ($LVED_d$) and left ventricular posterior wall thickness at systole ($PWT_s$) and diastole ($PWT_d$) were measured using the M-mode echocardiogram. Fractional shortening (FS) was calculated using $FS = (LVED_d - LVES_d) / LVED_d * 100$. Ejection fraction (EF) was calculated using the following equation: $EF = (LVED_d - LVES_d / LVED_d) * 100$. Stroke volume (SV) was calculated using the Doppler flow Velocity-Time Integral (VTI) at the LV outflow tract (LVOT) and the aortic diameter (Ao) ($LVOT^2 * 0.785 * Ao$ VTI). LV mass was calculated using the equation (left ventricular anterior wall (LVAW) + $LVED_d$ + $PWT_d)^3 - (LVED_d)^3$ [19].

### 2.5. Ventricular myocyte isolation and functional assessment

Following echocardiography, cardiomyocytes were isolated as previously described [3,18,20–23]. Briefly, the hearts were removed and retrograde perfusion was performed with buffer, followed by 0.25 mg/ml Liberase DH (Roche), 0.14 mg/ml 2.5% trypsin (Gibco) and 12.5 μM $CaCl_2$ for 5–7 min. Cells were dissociated by repeated pipetting and then filtered to remove cellular debris. Cardiomyocytes were exposed to increasing concentrations of $CaCl_2$ (to minimize the calcium paradox) and then plated on laminin-coated glass chambers.

Cardiomyocyte sarcomere function was examined using the Soft Edge MyoCam system (IonOptix Corporation), as previously described [3,18,20–23]. Only myocytes with normal sarcomeric appearance (absence of sarcomeric blebs) were used in these experiments. Peak shortening (sarcomere BL (baseline)% peak height; cellular equivalent of %FS), time to 90% of peak shortening (TPS90), time to 90% relengthening (TR 90), and velocities of shortening (−dL/dt) and relengthening (+dL/dt) were measured during stimulation at 1 Hz.

### 2.6. Assessment of cardiomyocyte calcium signaling

$Ca^{2+}$ transients and caffeine-induced $Ca^{2+}$ release were measured in isolated myocytes loaded with fura-2AM, as previously described [3,18,20–23]. Briefly, myocytes were incubated with 0.5 μM fura-2AM for 10 min and fluorescence was recorded using the Ionoptix dual-excitation fluorescence photomultiplier system through a 40× oil immersion objective. Fluorescence was achieved by excitation at 360 nm, followed by 380 nm, and emission was detected between 480–520 nm. For $Ca^{2+}$ transient measurements, cells were stimulated at 0.5 Hz and