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Short Communication

Metronomic Chemotherapy with Cisplatin Induces Skeletal Muscle Wasting and Impairs Ubiquitin-Proteasome System in B16-F10 Tumor-Bearing Mice

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Abstract

In this short communication, we provide evidence that metronomic chemotherapy with cisplatin induces skeletal muscle atrophy in B16-F10 tumor-bearing mice. Additionally, metronomic chemotherapy reduced 26S proteasome chymotrypsin-like activity and increased polyubiquitinated protein accumulation in *plantaris* muscle, but no differences were observed in *soleus* muscle. These findings indicate that cisplatin administration impairs protein quality control preferentially in muscles with high prevalence of glycolytic fibers. Based on these pre-clinical data, potential side effects of platinum-based chemotherapy on muscle physiology should be considered since skeletal muscle wasting is commonly associated with poor prognosis and lower life expectancy during cancer progression.

RESULTS AND DISCUSSION

Cisplatin is a chemotherapy agent used for treatment of many different cancer types in low and middle income countries and is an option for palliative care of patients with melanomas: the deadliest form of skin cancer. Cisplatin is cytotoxic and acts by stopping DNA replication and transcription, which results in cellular apoptosis [1,2]. Cisplatin treatment induces death in human melanoma SKmel37 cells *in vitro* and reduces tumor evolution when SKmel37 cells are injected in nude mice [3]. However, despite its efficacy, platinum-based agents have shown a broad spectrum of side effects, such as nausea, neuropathy, hepatotoxicity and nephrotoxicity [1,4]. Importantly, side effects were also reported in the skeletal muscle [5-8], including muscle atrophy. This is of particular importance since skeletal muscle wasting is associated with poor prognosis and lower life expectancy during cancer progression [9-11]. In this sense, metronomic chemotherapy has been proposed to attenuate side effects [12,13]. Metronomic chemotherapy consists of administrating drugs at relatively low doses without extended drug-free intervals that are characteristic of high dose chemotherapy. Therefore, this study aimed to evaluate the effects of both metronomic chemotherapy (*i.e.* daily low-dose cisplatin injections) and high-dose chemotherapy (*i.e.* three interposed injections of the maximal tolerated dose of cisplatin) in B16-F10 tumor-bearing mice, a murine model of melanoma.

Subcutaneous injection of B16-F10 tumor cells in mice has been previously proposed as a model of cancer cachexia [14,15]. Cancer cachexia is a multifactorial syndrome that involves skeletal muscle wasting associated with functional impairment and poor prognosis [10,16,17]. However, loss of body mass after B16-F10 injection in mice is not consistently observed in the literature (18). In the current study, we observed a clear reduction in body mass at 15 days post injection (dpi) of 2 x 10^5B16-F10 cells in absence of any change in *plantaris* and *soleus* muscle mass (Figures 1A-B). We speculated that B16-F10 tumor cell injection might induce loss of fat mass, but not skeletal muscle wasting in this subset of mice and at this time point. In accordance with this, previous studies have proposed that cancer-induced loss of fat mass precedes skeletal muscle wasting [14,15,19]. Thus, the effects induced by injection of B16-F10 cells seem time course-dependent in skeletal muscle.

Body mass and tumor growth were unchanged after both metronomic or high--dose chemotherapy (Figure 1A and C). Remarkably, metronomic chemotherapy, but not high-dose chemotherapy, induced *plantaris* muscle atrophy in B16-F10 tumor-bearing mice (Figure 1B). No significant differences were observed among all experimental groups for *soleus* muscle mass. *Plantaris* presents more type II fibers than *soleus* in mice [20-22]. Our data suggest that muscles with high prevalence of type II fibers are more susceptible to atrophy as a chemotherapy side

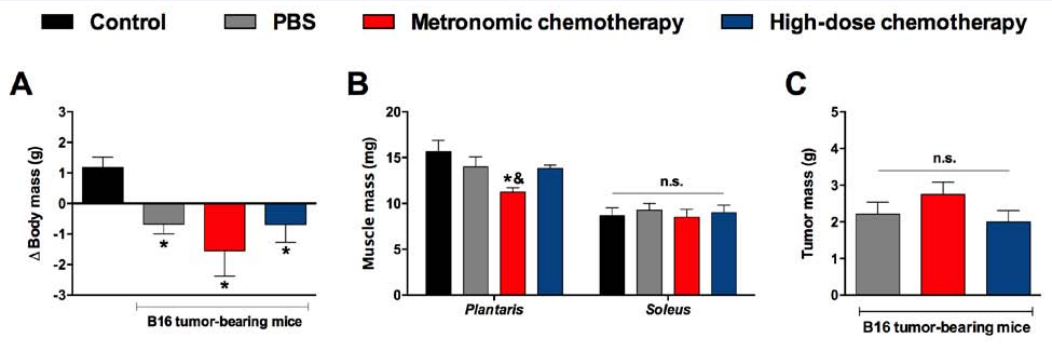

Figure 1 Metronomic chemotherapy with cisplatin induces *plantaris* muscle atrophy in B16-F10 tumor-bearing mice. Body mass delta changes (A), skeletal muscle mass (B) and tumor mass (C) in healthy control and B16-F10 tumor-bearing mice treated with PBS, metronomic chemotherapy (daily low-dose cisplatin injections) or high-dose chemotherapy (three interposed high-dose cisplatin injections). Data are presented as mean ± SEM. * indicates significant difference when compared to the Control group. & indicates significant difference when compared to the PBS group.n.s. indicates not significant difference in ANOVA. n = 3-5.

effect than muscles with predominantly of type I fibers. In line with these findings, some studies have demonstrated that type II fibers are more susceptible to cachectic stimuli [23,24]. This intrinsic protection of type I fibers has, at least in part, been attributed to higher innate oxidative and antioxidant capacities [25]. Cisplatin induces oxidative stress in the tumor microenvironment, but also in other tissues [26-29]. Thus, a putative mechanism that could explain the intrinsic protection of *soleus* muscle against cisplatin toxicity is the elevated intracellular antioxidant capacity.

We further investigated whether cisplatin administration could affect proteasome activity and protein ubiquitination. The ubiquitin-proteasome systemis a proteolytic downstream pathway responsible for removal of several damaged protein and plays a major role in cellular protein quality control [16,30-32]. Even though over expression of ubiquitin-proteasome system components has been associated with skeletal muscle wasting [10,31,33-38], we found that metronomic chemotherapy, but not high-dose chemotherapy, reduced 26S proteasome chymotryps in-like activity in *plantaris* muscle (Figure 2A). No significant differences were observed between any experimental groups for proteasome activity in *soleus* (Figure 2B). Additionally, polyubiquitinated proteins accumulated in *plantaris*, but remained unchanged in *soleus* after metronomic chemotherapy (Figures 2C and D). Since the 26S proteasome is implicated in the degradation of polyubiquitinated proteins, our findings suggest that the proteasome failed to breakdown the polyubiquitinated proteins, suggesting impairment in the protein quality control process. One potential explanation for changes in the ubiquitin-proteasome system is an accumulation of non-degradable protein aggregates that can inactivate the proteasome [30,39]. Notably, these data support previous concerns highlighted by our group regarding proteasome inhibition as a therapeutic target for skeletal muscle wasting conditions [31].

We acknowledge limitations in our study. Firstly, tumors weighed more than two grams at 15 dpi (Figure 1A), showing rapid tumor growth after injection of B16-F10 cells in mice. This fast tumor progression is a likely reason to explain the lack of chemotherapy effects in attenuating tumor growth. Further studies exploring cisplatin effects in other models with slower cancer progression are still necessary. Secondly, the present study design did not include healthy mice treated with chemotherapy, which limits our conclusions to B16-F10 tumor-bearing mice. Finally, we used a synthetic substrate for the proteasome activity assay, which does not allow identification of which proteins accumulated due to proteasome inactivity. Finally, we measured the 26S proteasome chymotrypsin-like catalytic site activity (*i.e.* the main proteolytic site involved in protein degradation), but we cannot exclude a potential compensatory participation of other proteasome catalytic sites, such as the caspase-like site [40]. Moreover, lysosomal proteolysis also should be considered in further exploratory studies.

In summary, this short communication presents evidence that metronomic chemotherapy with cisplatin induces skeletal muscle wasting and impairs the ubiquitin-proteasome systemin B16-F10 tumor-bearing mice. We found a reduction in 26S proteasome activity associated with higher polyubiquitinated protein levels during skeletal muscle wasting induced by cisplatin administration. Based on these pre-clinical data, potential side effects of platinum-based chemotherapy on muscle physiology should be considered and further studies are encouraged to explore the mechanisms underlying these side effects.

EXTENDED METHODS AND MATERIALS

Animals and study design

A set of eight-week-old male wild type C57BL/6mice were housed in an animal facility under controlled temperature (21°C) with 12:12 hours light: dark cycle. Animals had *ad libitum* access to standard laboratory chow and water. Mice were randomly assigned into four experimental groups: 1) healthy control mice treated daily with saline solution (Control; n = 3), 2) B16-F10 tumor-bearing mice treated daily with saline solution (PBS; n = 5), 3) B16-F10 tumor-bearing mice treated with daily low-dose cisplatin (metronomic chemotherapy; n = 5) and 4) B16-F10 tumor-bearing mice injected with interval high-doses of cisplatin (high-dosechemotherapy; n = 5). Body mass was assessed before (PRE) and 15 days post tumor cells injection (dpi). Mice were killed by cervical dislocation under anesthesia at 15 dpi. Tumors

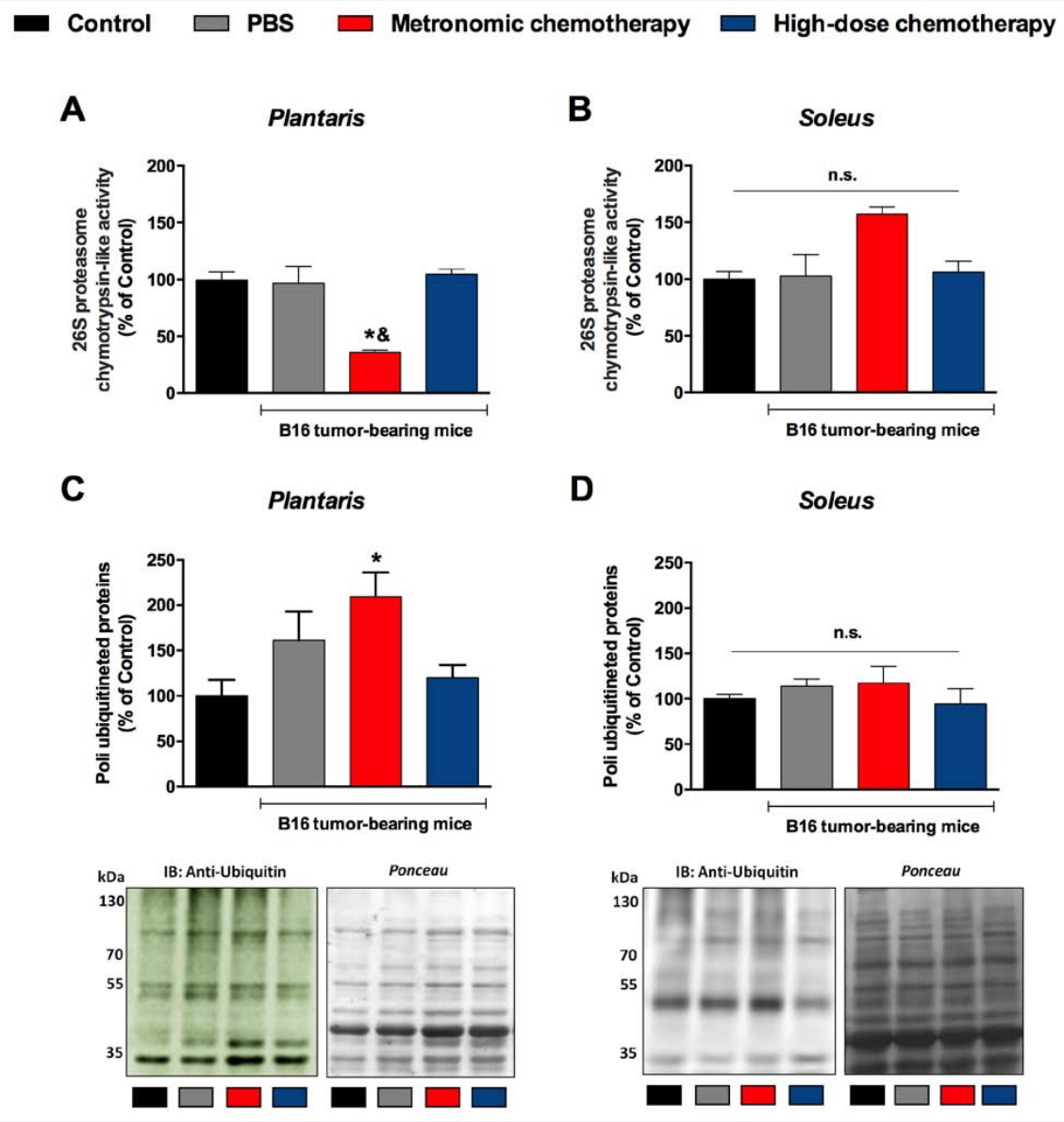

Figure 2 Metronomic chemotherapy with cisplatin reduces proteasome activity and increases protein ubiquitination in *plantaris*, but not in *soleus* muscle. 26S proteasome chymotrypsin-like activity in *plantaris* (A) and *soleus* (B) and protein ubiquitination in *plantaris* (C) and *soleus* (D) in healthy control and B16-F10 tumor-bearing mice treated with PBS, metronomic chemotherapy (daily low-dose cisplatin injections) or high-dose chemotherapy (three interposed high-dose cisplatin injections). Data are presented as mean ± SEM. * indicates significant difference when compared to the Control group. & indicates significant difference when compared to the PBS group.n.s. indicates not significant difference in ANOVA. n = 3-5.

were carefully harvested and weighted. Body mass delta changes (15 dpi – PRE) were calculated and tumor mass was discounted to estimate the carcass mass. *Plantaris* and *soleus* muscles were harvested, weighted, snap-frozen in liquid nitrogen and stored in a -80 freezer for further analyzes of proteasomal activity and protein ubiquitination. *Plantaris* and *soleus* were chosen due to the high prevalence of type II and I fibers, respectively, known to display different responses to cachectic stimuli [23,24]. All procedures were performed in accordance with the Guide for the Care and Use of Laboratory Animals (National Institutes of Health, USA) and with ethical principles in animal research adopted by the Brazilian College of Animal Experimentation (www.cobea.org.br). This study was approved by the Ethical Committee for Animal Research of School of Medicine, University of São Paulo, São Paulo, Brazil (protocol: 237/11).

Tumor cells injection and chemotherapy treatment

B16-F10 melanoma cell line (ATCC, USA) was cultured in RPMI-1640 (Gibco, NY, US) medium supplemented with 10% fetal bovine serum (Gibco, NY, US). Mice received a subcutaneous injection of 2 x 10^5 cells (diluted in 100 uL of serum-free RPMI-1640 medium) in the right flank. Chemotherapy treatment consisted of intraperitoneally (i.p.) injections with cisplatin (Sigma-Aldrich, MO, US) diluted in saline solution. Cisplatin treatment started at 7 dpi. Metronomic chemotherapy comprised

daily low-dose cisplatin (0.2 mg/kg of body mass), while high-dose chemotherapy comprised high-dose cisplatin (2.0 mg/kg of body mass) at 7, 10 and 13 dpi. Control and PBS experimental groups received daily i.p. injections with saline solution. Additionally, mice from high-dosechemotherapy group received daily injections with saline solution when cisplatin was not administered.

Proteasome activity

26S proteasome activity was measured by using a substrate for proteasome chymotrypsin-like catalytic site (Suc LLVY-AMC, Enzo Life Sciences, USA). Reaction mixture contained 25 mMTris (pH 7.4), 5 mM $MgCl_2$, 25 μM ATP, 25 μM LLVY-AMC and the sample (50 μg of soluble proteins for *plantaris* and 12.5 μg of soluble proteins for *soleus*). Fluorescent product formation was followed for 60 min (440 nm and 350 nm were emission and excitation wavelengths, respectively) at 37°C in the presence or absence of epoxomicin (20 μM), a highly-specific inhibitor of chymotrypsin-like proteasome activity, and the difference between the two rates was considered as 26S proteasomal activity (chymotrypsin-like site). Data are expressed as % of control group (set as 100%).

Protein ubiquitination

Polyubiquitinated protein levels were assessed in the *plantaris* and *soleus* muscle by western immunoblotting as previously described [41.42]. Primary antibody against ubiquitin (#sc8017, 264 Santa Cruz Biotechnology, Santa Cruz, CA, USA) was used. Targeted bands were normalized by Ponceau S staining (0.5% Ponceau S in 5% acetic acid). Protein ubiquitination was analyzed in a range from ˜30 to 130 kDa and data are expressed as % of control group (set as 100%).

Statistical analysis

Values are presented as means ± standard error of the mean (SEM). Analyses were conducted using Graph Pad Prism 6 (Graph Pad Software Inc., USA). One-way analysis of variance (ANOVA) was applied for all variables. Whenever significant effects were found, a Fisher's least significance difference (LSD) test was used for multiple comparison purposes. Statistical significance was set at $p < 0.05$.

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