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Mitochondrial and sarcoplasmic reticulum abnormalities in cancer cachexia: Altered energetic efficiency?

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ABSTRACT

Background: Cachexia is a wasting condition that manifests in several types of cancer, and the main characteristic is the profound loss of muscle mass.
Methods: The Yoshida AH-130 tumor model has been used and the samples have been analyzed using transmission electronic microscopy, real-time PCR and Western blot techniques.
Results: Using in vivo cancer cachectic model in rats, here we show that skeletal muscle loss is accompanied by fiber morphologic alterations such as mitochondrial disruption, dilatation of sarcoplasmic reticulum and apoptotic nuclei. Analyzing the expression of some factors related to proteolytic and thermogenic processes, we observed in tumor-bearing animals an increased expression of genes involved in proteolysis such as ubiquitin ligases Muscle Ring Finger 1 (MuRF-1) and Muscle Atrophy F-box protein (MAFBx). Moreover, an overexpression of both sarco/endoplasmic Ca^{2+}-ATPase (SERCA1) and adenine nucleotide translocator (ANT1), both factors related to cellular energetic efficiency, was observed. Tumor burden also leads to a marked decreased in muscle ATP content.
Conclusions: In addition to muscle proteolysis, other ATP-related pathways may have a key role in muscle wasting, both directly by increasing energetic inefficiency, and indirectly, by affecting the sarcoplasmic reticulum–mitochondrial assembly that is essential for muscle function and homeostasis.
General significance: The present study reports profound morphological changes in cancer cachectic muscle, which are visualized mainly in alterations in sarcoplasmic reticulum and mitochondria. These alterations are linked to pathways that can account for energy inefficiency associated with cancer cachexia.

1. Introduction

In advanced malignant diseases, cachexia appears to be one of the most common systemic manifestations. The presence of cachexia always implies a poor prognosis, having a great impact on the patients' quality of life and survival [1]. The skeletal muscle loss is the main characteristic of cancer cachexia and the principal cause of function impairment, fatigue and respiratory complications [2]. Several important molecular mechanisms have been shown to be involved in the increased muscle catabolism observed in cancer-induced cachexia, such as greater ubiquitin–proteasome-dependent proteolysis, apoptosis, and activation of uncoupling proteins [3]. Interaction of these mechanisms leads to muscle-mass loss by promoting protein and DNA breakdown and energy inefficiency.

Skeletal muscle is a very heterogeneous tissue and is used to respond to a broad range of functional demands in each animal species. It represents approximately 50% of the whole total weight and plays a central role in the whole-body metabolism [4]. Consequently, the loss of muscle in catabolic syndromes, such as cancer cachexia, represents a devastating condition not only for patient's quality of life, but also as a surgical risk and also decreasing the response to chemotherapy [3,5]. It is important to emphasize that approximately 30% of body weight loss represents a 75% of muscle loss. This factor leads to patient death, and it is the most prominent phenotypic feature in cancer cachexia [6]. In many years, the experts are challenging to understand what mechanisms are involved in the maintenance of muscle mass for the development of strategies to attenuate the wasting and improve muscle function [7].

It is known that mitochondria and sarcoplasmic reticulum (SR) have a key role in the muscular function. This advantageous assembly reflects the Ca^{2+} releasing from SR, stimulating mitochondrial ATP production helping to meet increased energy demand during muscle contraction, a process called excitation–contraction (EC) coupling [8]. On the other hand, functionally intact mitochondria inhibit undesired

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localized SR Ca^{2+} release by controlling the local redox environment of the calcium release units (for a review, see Ref. [8]). Thus, bidirectional SR–mitochondrial communication provides a powerful local control mechanism for integrating Ca^{2+} release/reuptake and ATP utilization during muscle contraction with ATP production and skeletal muscle bioenergetics [9].

Reduced thermodynamic efficiency will result in an increased weight loss. The laws of thermodynamics are silent on the existence of variable thermodynamic efficiency in metabolic processes. Therefore such variability can be related to differences in weight loss. An alteration of energy balance is the immediate cause of cachexia [10]. Although alterations of energy intake are often associated with cachexia, it has lately become clear that increased energy expenditure is the main cause of wasting associated with different types of pathological conditions, such as cancer, infections and chronic heart failure. Different types of molecular mechanisms contribute to involuntary body weight loss [11].

Taking into consideration that skeletal muscle loss is the most prominent characteristic of cancer cachexia, and that the mitochondria and SR have an important role in the muscle function and energetic metabolism [2], the purpose of this work has been to examine if a dysregulation of mitochondria and SR functions could be involved in the development of cancer cachexia in animals bearing cachectic tumors, analyzing the putative pathways involved in energy efficiency and homeostasis.

2. Material and methods

2.1. Animals

5 weeks old male Wistar rats (Interfauna, Barcelona, Spain) were maintained at 22 ± 2 °C with a regular light–dark cycle (light on from 08:00 a.m. to 08:00 p.m.) and had free access to food and water. The diet (Panlab, Barcelona, Spain) consists of 63.9% carbohydrate, 14.5% protein, and 4% fat (the residue was non-digestible material). Food intake was measured daily. All animal manipulations were conducted in accordance with the European Community guidelines for the use of laboratory animals.

2.2. Tumor inoculation

Rats were divided into two groups, namely controls (n = 6) and tumor hosts (n = 7). The latter received an intraperitoneal inoculum of 10^8 AH-130 Yoshida ascites hepatoma cells obtained from exponential tumors [12]. 8 days after tumor transplantation, the animals were weighed and anesthetized with an i.p. injection of ketamine/xylazine mixture (3:1) (Imalgene and Rompun respectively). The tumor was harvested from the peritoneal cavity, and its volume and cellularity were evaluated. Several tissues were rapidly excised, weighed, and frozen in liquid nitrogen except for the muscles processed for electron microscopy and histology (see below).

2.3. Histology, SDH staining and total activity

During rat sacrifice, the EDL muscles were rapidly excised and divided in two parts in the mid-belly region, half directly frozen in liquid nitrogen for the enzymatic activity, half mounted in OCT and frozen in melting isopentane for histology. Ten micrometers of transverse sections were cut on a cryostat and later stained for SDH (succinate dehydrogenase) incubating for 30 min at 37 °C with 1 mg/mL NTB (nitrotetrazolium blue chloride) and 27 mg/mL Na-succinate in PBS. Afterwards the slides were washed three times in PBS, mounted with glycerol and photographed at different magnifications. Fiber cross-sectional area (CSA) was determined on randomly chosen 100 individual fibers (for both oxidative and glycolytic ones) by the Image J software.

As for the total SDH activity, the muscles were homogenized (5 wt.%/vol) in ice-cold 150 mM NaCl, 10 mM KH_2PO_4, 0.1 mM EGTA, 2 times 30 s using a turrax device and centrifuged for 5 min at 800 ×*g*. The supernant was collected and total protein content measured using the BCA protein assay (Pierce, Thermo Fisher Scientific, Rockford, IL, USA). 50 μL of protein homogenate were incubated with 200 μL reaction buffer containing 10 mM Na-succinate, DCPIP 50 μg/mL, 10 mM phosphate buffer (pH 7.4), 2 mM KCN, 10 mM $CaCl_2$, 0.05% BSA. The absorbance at 600 nm was measured at t0, 3 min and 20 min after the addition of proteins. The rate of disappearance of the absorbance between 3 and 20 min was corrected for the total protein loaded and used to calculate the SDH content.

2.4. Transmission electronic microscopy

Muscle pieces of 1 mm^2 were removed under a stereomicroscope and transferred to glass vials filled with 2% parafomaldehyde and 2.5% glutaraldehyde in phosphate buffer. They kept in the fixative for 24 h at 4 °C. Then, they were washed with the same buffer and postfixed with 1% osmium tetroxide in the same buffer containing 0.8% potassium ferricyanide at 4 °C. Then the samples were dehydrated in acetone, infiltrated with Epon resin for 2 days, embedded in the same resin orientated for longitudinal sectioning and polymerized at 60 °C during 48 h. Semithin sections were made in order to look for muscle fibers at light microscope. When they were found, ultrathin sections were obtained using a Leica Ultracut UC6 ultramicrotome and mounted on Formvar-coated copper grids. They were staining with 2% uranyl acetate in water and lead citrate. Then, sections were observed in a JEM-1010 electron microscope (Jeol, Japan) equipped with a CCD camera SIS Megaview III. The images were digitized and analyzed through the software AnalySIS (Soft Imaging System, Germany). Intermyofibrillar mitochondrial morphology was classified into unchanged and altered (swelling-related ultrastructural changes). Mitochondrial counting was performed from 25 to 30 micrographs, which were randomly taken fields at 20,000×–30,000× magnification, from three different areas of one grid.

2.5. ATP measurement

The determination of ATP using bioluminescence was performed using the commercially available kit ATP Bioluminescence Assay Kit CLS II (Roche) according to manufacturer's recommendations. Briefly, GSN muscles were homogenized in PBS (proportion 1:10 w/v). Then, the samples were diluted 10× in 100 mM Tris, 4 mM EDTA (pH 7.75), incubated 2 min at 100 °C and centrifuged 1 min at 1000 ×*g*. The supernatant was transferred to a new tube. In a multiwell black plate (96 wells — Packard) 50 μL of the sample and 50 μL of the Luciferase reagent were added. The luminescence was measured in a Luminometer at 562 nm with an integration time of 10 s. The ATP concentrations were obtained from a log–log plot of the standard curve data.

2.6. Real-time PCR (polymerase chain reaction)

Total RNA was extracted by using Tripure Isolation Reagent (Roche Applied Science, Switzerland) according to manufacturer's recommendations. Expression of *SERCA1* (5′-TTG TCC TAT TTC GGG GTG AG-3′ and 5′-TCC CAC AGA GAC TTG CCT TC-3′), *SERCA2* (5′-GCT TGT CCA TGT CCC TTC AC-3′ and 5′-ACT CCA GTA TTG CAG GCT CC-3′), *ANT1* (5′-GCT GGT GTC CTA TCC GTT TG-3′ and 5′-CAG TCA AGT GTC CCC GTG TA-3′), *RyR1* (5′-GTC TCT GTC AGT TCG AGC CC-3′ and 5′-GCC AAC TTG TCA GTC ATG GA-3′), *MFN2* (5′-GAG AGG CGA TTT GAG GAG TG-3′ and 5′-GTC AAT GAA TCT CAG CCG GT-3′), *CALP1* (5′-GAG GAA GAT GGG TGA GGA CA-3′ and 5′-GCT GAG GTG GAT GTT GGT CT-3′), *PGC-1a* (5′-AAG GTC CCC AGG CAG TAG AT-3′ and 5′-TCA GAC TCC CGC TTC TCA T-3′), *UB* (5′-CAC CAA GAA GGT CAA ACA GGA-3′ and