Free Radical Biology and Medicine 91 (2016) 204–214

Contents lists available at ScienceDirect

Free Radical Biology and Medicine

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

Original Contribution

Antioxidant supplementation accelerates cachexia development by promoting tumor growth in C26 tumor-bearing mice

Mohamad Assi [1], Frédéric Derbré [1], Luz Lefeuvre-Orfila, Amélie Rébillard *

EA1274 Laboratory "Movement, Sport and Health Sciences" M2S, University of Rennes 2-ENS Rennes, Bruz, France

ARTICLE INFO

Article history:
Received 24 October 2015
Received in revised form
14 December 2015
Accepted 16 December 2015
Available online 18 December 2015

Keywords:
Muscle wasting
TNF-α
Myostatin
Tumor growth
Oxidative stress

ABSTRACT

More than 50% of patients with advanced stages of colon cancer suffer from progressive loss of skeletal muscle, called cachexia, resulting in reduced quality of life and shortened survival. It is becoming evident that reactive oxygen species (ROS) regulate pathways controlling skeletal muscle atrophy. Herein we tested the hypothesis that antioxidant supplementation could prevent skeletal muscle atrophy in a model of cachectic Colon 26 (C26) tumor-bearing mice. Seven-week-old BALB/c mice were subcutaneously inoculated with colon 26 (C26) cancer cells or PBS. Then C26-mice were daily gavaged during 22 days either with PBS (vehicle) or an antioxidant cocktail whose composition is close to that of commercial dietary antioxidant supplements (rich in catechins, quercetin and vitamin C). We found that antioxidants enhanced weight loss and caused premature death of mice. Antioxidants supplementation failed to prevent (i) the increase in plasma TNF-α levels and systemic oxidative damage, (ii) skeletal muscle atrophy and (iii) activation of the ubiquitin-proteasome system (MuRF-1, MAFbx and poly-ubiquitinated proteins). Accordingly, immunohistological staining for Ki-67 and the expression of cell cycle inhibitors demonstrated that tumor of supplemented mice developed faster with a concomitant decrease in oxidative damage. Previous studies have shown that the use of catechins and quercetin separately can improve the musculoskeletal function in cachectic animals. However, our results indicate that the combination of these antioxidants reduced survival and enhanced cachexia in C26-mice.

1. Introduction

Cachexia is a multifactorial syndrome and a complex metabolic disorder that manifests mainly in patients with diabetes, acquired immune deficiency syndrome (AIDS) and cancer. It is characterized by a continuous loss of muscle mass with or without depletion of adipose tissue [1]. Contrary to anorexia, in which adipose tissue is mainly affected but rarely the muscle compartment, conventional and single-nutritional intervention failed to prevent total weight loss and/or restore lean body mass in cancer cachexia [2]. Patients with advanced stages of colon cancer are mainly affected by cachexia, which accounts for more than 20% of mortality in total cancer patients [3]. Muscle atrophy is one of the most significant clinical events in cancer cachexia that negatively impact patient's quality of life and strongly reduce anti-cancer treatment efficiency [4]. Cachexia-induced muscle atrophy (CIMA) is often associated with psychosocial effects due to functional impairment and a dramatically reduced physical activity in cachectic patients [5]. Therefore, the management of cancer cachexia, notably muscle atrophy, represents a real challenge and a major issue for researchers and clinicians in the biomedical field.

Disease-related muscle atrophy have received much attention during the past decade and several studies have been devoted to understand the pathophysiology and explore the underlying molecular mechanisms involved in the wasting process. Muscle atrophy is mainly due to an increase in protein catabolism associated with hypo-anabolism, resulting in a net decrease in overall muscle mass [6]. High circulating levels of tumor-derived factors (e.g. Tumor Necrosis Factor-α and Interleukin-6) and some Transforming Growth Factor-beta (TGF-β) family ligands (e.g. myostatin and activin), are responsible for the inhibition of the anabolic/hypertrophic, Phosphoinositide 3-kinase (PI3K)-Akt pathway and subsequently the depression in protein synthesis rate [7,8]. On the other hand, these cytokines, trigger the activation of catabolic/atrophic upstream signaling cascades (e.g. Nuclear Factor-κB/AP-1), leading to the transcriptional activation of two muscle-specific E3 ubiquitin-ligases, known as, Muscle Ring finger protein-1 (MuRF-1) and Muscle Atrophy F-box (MAFbx) [9,10]. Sarcomeric proteins destined to degradation are tagged with polyubiquitin chain, thanks to MuRF-1 and MAFbx, prior the entry into the proteasome core, where they undergo degradation into

* Corresponding author.
E-mail address: amelie.rebillard@univ-rennes2.fr (A. Rébillard).
[1] Co-first authors who contributed equally to this study.

http://dx.doi.org/10.1016/j.freeradbiomed.2015.12.019
0891-5849/

small peptide fragments. Hyper-activation of the ubiquitin-proteasome proteolytic system is common in CIMA and is mainly responsible for the high breakdown of myofibrillar proteins [11].

Currently, it is well established and recognized that muscle wasting is intimately linked to oxidative stress (OS) in various conditions including disuse, ageing and spinal cord injury [12,13]. It seems that OS is also implicated in cachexia pathogenesis, since oxidative damage markers (i.e. carbonyls proteins) were increased in the skeletal muscle of cachectic patients with lung cancer and were positively correlated with muscle proteolysis [14]. Furthermore, Reactive Oxygen Species (ROS) have been proposed to act as a second messenger to activate the ubiquitin-proteasome system in an *in vitro* model of muscle atrophy [15]. Actually, there is growing evidence that sarcopenia or disuse-induced catabolic state in the muscles of rodents models, could be prevented or delayed by the use of different types and doses of antioxidants such as Vitamin E, Resveratrol, Cystein-based antioxidants and Allopurinol [16–19]. Since a broad range of ROS is involved in cachexia-related muscle atrophy pathogenesis, we suppose that the use of a combination of antioxidants could be more valuable to target a large spectrum of these species and limit their detrimental activities. Herein, we tested our hypothesis in a model of colon 26 (C26) tumor-bearing mice supplemented or not with a mixture of antioxidants containing mainly, catechins, curcumin, quercetin and vitamin C.

2. Materials and methods

2.1. Animals care and protocol

All experiments were approved by the Regional Ethics Committee for Animal Experimentation of Brittany (No. R-2012-AR-01) and conducted in accordance with the current ethical standards of the European Community (Directive 2010/63/EU). Seven-week-old male Balb/c mice were obtained from Janvier Labs (Le Genest Saint Isle, France) and housed in the Animal Care Facility of the laboratory "Movement, Sport and health Sciences" accredited for live animal experimentation (No. A35-047-34, delivered by the Department of Veterinary Services, French Ministry of Agriculture). Mice were maintained on a 12:12-h dark–light cycle. Water was available ad libitum and standard laboratory chow was provided, changed and monitored daily. The animals were divided into three experimental groups: control group that received a daily supplement of 250 µl of PBS ($n=10$); C26 tumor-bearing group that received 250 µl of PBS ($n=10$); and C26 tumor-bearing group that was daily supplemented with an antioxidant mixture rich in catechins, curcumin, quercetin and vitamin C (81.5 mg/kg of body weight) dissolved in 350–500 µl of PBS ($n=10$). The volume of the daily supplement received by each mouse in this group was daily adjusted with body weight change. All treatments were administrated by oral gavage. The composition of the antioxidant cocktail is presented in Table 1. Twenty-two days after inoculation of C26 cells or when mice have lost more than 20% of body weight, animals were anesthetized with a ketamine–xylazine–butorphanol cocktail. *Soleus*, *Extensor digitorum longus* (EDL), *rectus femoris*, *gastrocnemius* (Gas) muscles and tumor tissues were weighed and immediately frozen in liquid nitrogen or fixed in 4%-paraformaldehyde (PFA). Venous blood was collected into EDTA tubes and centrifuged (1500*g*, 10 min, 4 °C) for plasma collection.

2.2. Culture of C26 cell line and inoculation

Frozen C26 colon cancer cells were obtained from Cell Lines Service (CLS, Eppelheim, Germany). The murine tumor cells were maintained in a humidified atmosphere at 37 °C and 5% CO_2 and cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine (Life technologies, Saint Aubin, France). Cells were collected using 0.25% trypsin-EDTA. Before the injection of C26 cells into mice (day 1), cells were counted using a hemocytometer, pelleted via centrifugation (500*g*, 5 min, 25 °C) and resuspended at 1×10^6 C26 cells/mL of sterilized PBS. Mice were shaved on the dorsal side and given a subcutaneous injection of either 1×10^6 C26 cells suspended in 100 µl of sterilized PBS or 100 µl of sterilized PBS only (control) [20,21]. Body mass was monitored daily after inoculation.

Table 1
Composition of antioxidant mixture.

	Nutritional information of antioxidant cocktail (mg/kg/day)
Green tea extracts	11.53
Included polyphenols	3.46
Curcumin	8.65
Quercetin	4.33
A-lipoic acid	2.88
Oligomeric procyanidins	2.88
Lutein	0.58
Piperin	0.29
Resveratrol	0.14
Zinc	1.08
Manganese	0.14
Selenium	0.0036
Vitamin A	0.06
Vitamin C	11.53
Vitamin E	1.73
Vitamin B1	0.16
Vitamin B2	0.20
Vitamin B3	2.31

2.3. Determination of cachexia symptoms severity

Mice were classified as having mild, moderate, or severe cachexia based on their body mass and *gastrocnemius* muscle mass. C26 tumor-bearing mice were categorized as having mild cachexia, if both variables were within 1 SD (Standard Deviation) of the mean of control mice. Mice with severe cachexia were >2 SDs away from the mean of control mice [22].

2.4. Wire hang test

At the beginning of the protocol, 10 days and 22 days after inoculation, a wire test was used to assess whole body force. Mice were placed on top of a wire cage lid that was shaken gently 3 times, causing the mouse to grip the wire. Then, the wire cage lid was inverted. Three trials per animal were performed for each evaluation and the best time of latency was retained to calculate the holding impulse [23].

2.5. Histological analysis

EDL muscle or tumor samples were 24 h PFA-fixed and paraffin-embedded in blocks. Serial transverse sections of 4 µm were obtained from each sample using a LEICA microtome and were mounted on glass slides, three cuts in each. The cuts were performed in the wider part of the tissues. Subsequently, EDL sections were stained for reticulin using the Gomori method to measure muscle fibers diameter and tumor sections were stained for Ki-67 in order to determine the percentage of tumor proliferating cells. All the histological sections were scanned (20× objective and 40× magnification). The minimal Feret's diameter of muscle fiber was determined using the Image J software. Mean fiber diameter for each muscle was determined from at least 150 fibers per