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L-leucine dietary supplementation modulates muscle protein degradation and increases pro-inflammatory cytokines in tumour-bearing rats

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ABSTRACT

Cancer cachexia is characterised by involuntary weight loss associated with systemic inflammation and metabolic changes. Studies aimed at maintaining lean body mass in cachectic tumour-bearing hosts have made important contributions reducing the number of deaths and improving the quality of life. In recent years, leucine has demonstrated effective action in maintaining lean body mass by decreasing muscle protein degradation. Currently, there is a growing need to understand how leucine stimulates protein synthesis and acts protectively in a cachectic organism. Thus, this study aimed to assess the effects of a leucine-rich diet on protein degradation signalling in muscle over the course of tumour growth. Animals were distributed into four experimental groups, which did or did not receive 2×10^6 viable Walker-tumour cells. Some were fed a leucine-rich diet, and the groups were subsequently sacrificed at three different time points of tumour evolution (7th, 14th, and 21st days). Protein degradation signals, as indicated by ubiquitin-proteasome subunits (11S, 19S, and 20S) and pro- and anti-inflammatory cytokines, were analysed in all experimental groups. In tumour-bearing animals without nutritional supplementation (W7, W14, and W21 groups), we observed that the tumour growth promoted a concurrent decrease in muscle protein, a sharp increase in pro-inflammatory cytokines (TNFα, IL-6, and IFNγ), and a progressive increase in proteasome subunits (19S and 20S). Thus, the leucine-supplemented tumour-bearing groups showed improvements in muscle mass and protein content, and in this specific situation, the leucine-rich diet led to an increase on the day in cytokine profile and proteasome subunits mainly on the 14th day, which subsequently had a modulating effect on tumour growth on the 21st day. These results indicate that the presence of leucine in the diet may modulate important aspects of the proteasomal pathway in cancer cachexia and may prevent muscle wasting due to the decrease in the cachexia index.

1. Introduction

Cachexia is one of the most important effects of some types of cancer. Among other symptoms, involuntary weight loss and malnutrition are the most common cachectic characteristics observed in cancer patients. This syndrome leads to intense host tissue wasting and subsequent intense body weight loss, especially due to the loss of adipose tissue and muscle mass [1]. Walker 256 rat carcinoma cells originated from a tumour that produced effects resembling those of various cachectic cancers; therefore, these cells are extensively used to establish experimental models of cachexia in rats [2].

Many studies have indicated that the most important goal during cancer progression is the maintenance of lean body mass to improve the prognosis, reduce death, and maintain cancer patients' quality of life. In recent years, leucine has demonstrated efficacy in maintaining lean body mass by stimulating muscle protein synthesis. Among the branched-chain amino acids (BCAA), leucine assumes an important role in the regulation of protein metabolism. *In vivo* and *in vitro* studies have corroborated the fact that a diet supplemented with leucine stimulates protein synthesis, especially in skeletal muscle [3–6]. Similarly, other studies have indicated that leucine supplementation may even reverse or minimise protein catabolism [7,8].

Only a few studies are related to alterations in the tumour-bearing organism throughout tumour evolution. Usually, the literature presents studies that have analysed only one-time point for tumour evolution (for example, rats in the pre-agonic stage). A relevant issue in the present work is the point at which nutritional supplementation begins to prevent/lessen the onset of tumour effects. Therefore, studying the modulatory effects caused by leucine supplementation over time course experiments was beneficial for helping to define the point at which nutritional supplementation began to exert its benefits on lessening tumour effects or improving host responses.

The modulation of pro- and anti-inflammatory interleukins is an important characteristic of the immune system during the pathological

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state [9]. Interleukin levels are changed in tumour-bearing organisms in order to prevent/lessen tumour growth [10–12]. Nutritional supplementation has a role in fostering cytokine action in the tumour-bearing host, thus indirectly modulating tumour effects [13].

Currently, our understanding regarding the mechanism by which leucine imparts a protective effect in skeletal muscle must be expanded in order to better comprehend the reversion of protein catabolism. Therefore, the main aim of this study was to assess leucine effects in relation to the time course of tumour progression by assessing various proteins critical to the proteolytic process in gastrocnemius muscle tissue in Walker 256 tumour-bearing rats.

2. Materials and methods

2.1. Diets

The semi-purified isocaloric diets included normal protein (C), 18% protein (AIN-93G) [14] and leucine (L), or 18% protein plus 3% L-leucine. The diets were supplemented with approximately 70% carbohydrates (sucrose, dextrin, and starch), 7% fat (soybean oil), and 5% fibre (purified micro-cellulose) in addition to vitamin and mineral mixes, cysteine, and choline. The control diet contained 1.6% L-leucine, whereas the leucine-rich diet contained 4.6% L-leucine as based on previous experimental studies [8,15].

2.2. Animals

Female Wistar rats (n = 72 animals; 90 days old; weighing 180–200 g) were obtained from the animal facilities centre at UNICAMP (CEMIB/State University of Campinas, Brazil), and the rats received food and water ad libitum under a 12-h light-dark cycle and constant temperature (22 ± 2 °C) and humidity (50–60%). The choose to use female rats was based on our previous research [7,16–19] which showed that during tumour development the female reproductive cycle stopped at the diestrus stage and did not affect the results acquired in this kind of experimental tumour model. The animals were distributed into 12 groups according to the inoculation of Walker 256 tumour cells (1×10^6 viable tumour cells counted by trypan blue exclusion), the tumour growth time points (7, 14, and 21 days after inoculation) and the provision of a leucine-rich diet. Each group contained six animals, which were treated according to Table 1.

The rats remained in collective cages throughout the experimental period, and groups of animals were sacrificed 7, 14, or 21 days after tumour cell inoculation to evaluate and establish trends in the protein degradation-associated cell signalling profiles. The gastrocnemius muscles were then dissected; portions were weighed, quickly frozen in liquid nitrogen, and then stored in a bio-freezer for further biochemical and molecular analyses. The general United Kingdom Coordinating Committee on Cancer Research guidelines for animal welfare were followed, and the institutional committee approved the protocols based on ethical standards in animal research (CEEA/IB/UNICAMP, protocol number #2418-1) [20].

2.3. Western blot assay

The gastrocnemius muscles were homogenised with homogenisation buffer (20 mM Tris, 1 mM dithiothreitol (DTT), 2 mM adenosine triphosphate (ATP) and 5 mM $MgCl_2$), centrifuged, and divided into aliquots for the analysis of total protein [21]. We also verified the expression of various key proteins of the ubiquitin-proteasome system (proteasome subunits 20Sα, 19S, and 11S; Affinity, USA; diluted 1:1500) in the muscle. In total, 2.5 μg of muscle homogenate protein was resolved by sodium dodecyl sulphate polyacrylamide gel electrophoresis (12%) and transferred to a 0.45-μm pore size nitrocellulose membrane. The expression of ubiquitin-proteasome system proteins was assessed with antibodies against the following subunits: 20Sα (Affinity USA, 1:1000 dilution), 19S-dependent proteasome unit (ATP, Affinity USA, 1:1500 dilution) and 11S subunit (Affinity USA, 1:1000 dilution). The presence of these subunits was determined using the following secondary antibodies: anti-rabbit, anti-mouse and anti-goat, respectively. The expression of these proteins was standardised using GAPDH as a loading control. Enhanced chemiluminescence (Amersham GE Healthcare, USA) was used to visualise the bands. The densitometry analysis of the protein bands was performed using Image Capture (Amersham GE Healthcare) and Gel Pro II software.

2.4. Serum cytokine analysis

Several cytokines (IL-4, IL-6, IL-10, TNF, and IFNγ) were analysed using multiplex kits (Multiplex Kit, Millipore, USA) with coupling beads and cytokine-specific capture antibodies. Plasma samples previously prepared with antibody conjugated-beads against the cytokines were aliquoted into a multiwell plate. The plate was stirred overnight at 4 °C and washed twice with sheath fluid buffer supplied by the manufacturer (Millipore, USA) to remove material not bound to the beads. Following the addition of the detection antibodies, the plate was stirred for 2 h at room temperature, 24 °C. Phycoerythrin-conjugated streptavidin was then added, and the plate was further incubated for 30 min. Fluorescence was measured using a Luminex®200™ system (Luminex Corporation, TX, USA). The analyses performed with xPonent® 3.1 Software (Luminex Corporation, TX, USA) provided by the Luminex®200™ system.

2.5. Statistical analysis

The results are expressed as the mean ± standard error of the mean, using the Graph Pad Prism 6.0 software (Graph-Pad Software, Inc.). For comparisons among multiple groups (such as C, W, L, and LW), data were evaluated with analysis of variance (two-way ANOVA) to determine the effects of diet and/or tumour growth on all parameters, followed by post hoc comparison using Bonferroni's test. P values < 0.05 were considered significant [22].

3. Results

3.1. Body weight and tumour development

The body weight of the animals increased in the leucine-treated groups. This increase was approximately 2.5% in the 7L group and became significant on the 14th and 21st days at approximately 6.5% in the 14L group and 7% in the 21L group when compared with the respective 7C, 14C, and 21C groups (Fig. 1A and C). These data showed that diet accounted for 9.3% of the total variance and influenced the whole body weight evolution ($P = 0.0118$). Among the tumour-bearing groups, the W group had a significant decrease in body weight evolution; despite having reductions in body weight, the leucine group (7WL, 14WL, and 21 WL) showed an increase in body weight compared to W groups (Fig. 1A). The body weight gain (Fig. 1C) showed that tumour effects accounted for 66% of total variance and reduced the body weight in tumour-bearing groups compared to C and L groups ($P < 0.0001$). The interaction effect, however, showed that diet accounted for 26% of the total variance and modulated tumour effects preferentially over body weight in WL group ($P = 0.0432$). Despite the nutritional differences, no differences in tumour development were noted between the W and WL groups; the final tumour weight was 37.28 g ± 3.51 g in the 21 W group and 40.46 g ± 3.31 g in the 21 WL group (Fig. 1B). This type of tumour represents an experimental model of cachexia, and a 10% tumour-to-body weight ratio indicates that the host is suffering from the effects of cachexia (Fig. 1B). To assess cachexia, we used the cachexia index [19,23], which corresponds to the percentage of the initial body weight minus the carcass mass plus tumour weight added to the body mass gain of the control group