Dose-dependent effects of leucine supplementation on preservation of muscle mass in cancer cachectic mice

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Abstract. Cancer cachexia, which is characterized by muscle wasting, is associated with increased morbidity and mortality. Because muscle protein synthesis may be increased and protein breakdown reduced by leucine supplementation, we used the C26 tumor-bearing cachetic mouse model to assess the effects of dietary supplementation with leucine on muscle weight and the markers of muscle protein breakdown (mRNA of atrogin and murf). Male C2D2FI mice were subcutaneously inoculated with tumor cells (tumor-bearing mice; TB) or were sham injected (control; C). They were fed standard diets or diets supplemented with leucine [1 gr (TB1Leu) or 8 gr (TB8Leu) supplemented leucine per kg feed]; TB and C received 8.7% Leu/g protein, TB1Leu received 9.6% Leu/g protein and TB8Leu received 14.6 Leu/g protein. After 21 days, the following were determined: body weights, plasma amino-acid concentrations, tumor size and muscle mass of the gastrocnemius (mG), tibialis anterior (mTA), extensor digitorum longus (mEDL) and soleus (mS) muscles. In tumor-bearing (TB) mice, carcass and skeletal muscle masses decreased, and levels of atrogin and murf mRNA in the mEDL increased. Muscle-mass loss was counteracted dose-dependently by leucine supplementation: relative to TB, the mass of the mG was +23% in TB8Leu, and +22% in mTA (p<0.05). However, leucine supplementation did not change atrogin and murf mRNA levels. Total plasma amino acid concentrations increased in TB, especially for taurine, lysine, arginine and alanine (p<0.05). Leucine supplementation attenuated the increase in total plasma amino-acid concentrations (p<0.05). Irrespective of changes in muscle protein breakdown markers, leucine supplementation reduced muscle wasting in tumor-bearing cachetic mice and attenuated changes in plasma amino acids.

Introduction

Cancer cachexia is characterized by a progressive loss of fat mass and skeletal muscle mass. Because it results from systemic inflammation induced by the tumor (1-3), the metabolic changes it causes are different from those of starvation (4). The skeletal muscle wasting that accompanies cancer cachexia is associated with increased morbidity and mortality (4,5); its severity is inversely related to the length of time a patient survives (6). The skeletal muscle protein balance depends on the sum of protein synthesis and breakdown. Reportedly, protein synthesis is stimulated and protein breakdown is inhibited by the essential amino acid, leucine (7-10). In weight-losing cachetic cancer patients, the cause of muscle wasting is a decrease skeletal muscle-protein synthesis (11) or a lack to increase skeletal muscle-protein synthesis to counter-balance an increase in protein degradation (12). Net muscle-protein synthesis in these patients might thus be stimulated by a nutritional intervention with leucine, such as that used in...
tumor-bearing cachectic rats, where a diet containing 17 vs. 9% leucine altered proteasome activity (13) and reduced the loss of lean body mass, gastrocnemius mass and myosin content of skeletal muscle (10). This hypothesis is further supported by findings in diabetic and somatostatin-treated fasted rats, in which oral leucine at 1.35 g/kg BW enhanced skeletal-muscle protein synthesis via both insulin-dependent and insulin-independent mechanisms (14,15).

In this study, we tested the effect of leucine in the C26 cancer cachectic model, in which CD2F1 mice inoculated subcutaneously with murine colon adenocarcinoma (C26) cells (16-19) started to lose body weight after ~14 days, and became anorectic at days 19-20. In this model the animals lose more muscle, and therefore the window of possible effect is larger than in a similar but milder cancer cachectic model with no anorexia present as described previously by our group. In this model we observed that a dietary intervention combining fish oil, high protein and leucine had a synergistic effect on the maintenance of body composition (20,21). While the individual components preserved neither fat nor muscle mass, the combination preserved them both. Equally importantly, muscle function, daily activity and immune function all improved when specific oligosaccharides were combined with fish oil, high protein and leucine (20,21). In this study, in which leucine was found as a signal component to have a small but significant effect, we investigated the mechanisms and pathways behind the effect of leucine supplementation on cancer-cachexia-related muscle-mass loss.

Materials and methods

Animals. Male CD2F1 mice aged 6-7 weeks (BALB/c x DBA/2, Harlan/Charles River, Horst, The Netherlands) were individually housed in a climate-controlled room (12:12 dark-light cycle with a constant room temperature of 21±1°C). After acclimatization for one week, they were divided into weight-matched groups: i) a control group (C) that received control chow [AIN93M (22) containing 8.7% Leu per g of protein]; ii) a tumor-bearing group (TB) that received control chow; iii) a tumor-bearing group (TB1Leu) that received low leucine (AIN93M + 1 g leucine per kg feed, containing 9.6% Leu per g protein); and iv) a tumor-bearing group (TB8Leu) that received high leucine (AIN93M + 8 g leucine per kg feed, containing 14.8% leucine per g protein). Per kg feed, the AIN93M control diet contained 126 g protein (100% casein), 727 g carbohydrates, and 40 g fat (100% soy oil) (Research Diet Services, Wijk bij Duurstede, The Netherlands). All experimental procedures were approved by the Animal Ethics Committee (DEC consult, The Netherlands), and complied with the principles of good laboratory animal care.

Tumor model. Murine C-26 adenocarcinoma cells were cultured in vitro with RPMI-1640 (Life Technologies, Merelbeke, Belgium) supplemented with 5% fetal calf serum and 1% penicillin-streptomycin (16). Tumor cells were trypsinized in a sub-confluent state, and, after washing, suspended in Hank's balanced salt solution (HBSS) (Life Technologies) at a concentration of 2.5x10⁶ cells/ml. When the mice were under general anesthesia (isoflurane/ N₂O/O₂), tumor cells (1x10⁶ cells in 0.2 ml) were inoculated subcutaneously into the right inguinal flank. Control (C) animals received a sham injection with 0.2 ml HBSS. After inoculation of tumor cells or sham treatment, body mass, food intake and tumor size (length and width) were assessed three times per week.

At day 21 after tumor inoculation, the animals were anesthetized and blood was sampled by heart puncture. At section, the tumor and the skeletal muscles mEDL (extensor digitorum longus), mG (gastrocnemius), mS (soleus) and nTA (tibialis) were dissected and weighed.

HPLC analysis of plasma amino acids. To determine plasma 3-methylhistidine and the concentrations of amino acid in plasma and feed, we used HPLC with ortho-Phthalaldehyde as derivatization reagent and L-norvaline as internal standard (both from Sigma Aldrich). The method was adapted from van Eijk et al (23,24).

RNA isolation. Total RNA from EDL skeletal muscle tissue was isolated using the RNeasy kit (Qiagen Benelux BV., Venlo, the Netherlands) according to the manufacturer's instructions. Briefly, a minimum of 300 μl of lysis buffer was used to homogenize the muscle using a low-volume glass potter. After complete lysis, the lysate was diluted and subjected to a protease K (20 mg/ml) treatment. Debris was pelleted by centrifugation. Ethanol was added to the cleared lysate. RNA was bound to the silicagel membrane, and traces of genomic DNA were removed by DNase I treatment of the RNeasy column. After the column had been washed, the RNA was eluted in 30 μl of RNase-free water.

Total RNA quantification and real-time PCR. The total RNA content was measured using the procedure for the Ribogreen RNA quantitation kit (Invitrogen, Leiden, the Netherlands) outlined in the manufacturer's instructions, using the RNA supplied as a standard. Approximately 500 ng of total RNA were transcribed into cDNA using 100 ng/μl of random hexamers (Roche Diagnostics, Almere, The Netherlands) and M-MLV (Moloney Murine Leukemia Virus) reverse transcriptase (Invitrogen). Real-time PCR experiments were carried out in a 25 μl reaction comprised of TaqMan Universal Mastermix (TUM, Applied Biosystems, Nieuwekerk a/d IJssel, The Netherlands), 5 μl of cDNA, 900 nm of each forward and reverse primer, and 200 nm probe. A comparative Ct method was used to obtain a relative quantification of gene expression. GAPDH was used as a reference gene.

Statistical analysis. The data are expressed as the means ± SEM. Statistical analyses were performed using SPSS 15.0 (SPSS Benelux, Gorinchem, The Netherlands). Data from the different groups were compared with TB with one-way analysis of variance (ANOVA) and post-hoc LSD.

Results

Body mass, tumor weight and food intake. On day 21 after tumor inoculation, tumor-bearing mice (TB) had lower body and carcass weights (carcass weight = body weight - tumor weight) than control mice (Table 1A). Twenty-one days after tumor inoculation, leucine supplementation had not affected...
Body weight; neither had it affected tumor weight. On day 20, food intake was significantly lower in the TB group than in the C group. The food intake between tumor-bearing groups did not differ (Table IB).

HPLC determination showed that leucine concentrations in the feeds were 8.7% w/w protein for C and TB, 9.6% w/w in the low-leucine group (TB1leu), and 14.8% w/w in the high-leucine group (TB8leu). On day 0, the mice had an average weight of 23 g. On average, they consumed 4 g chow per day. Therefore, total daily leucine intake was 348 mg in the C and TB groups, 384 mg in the TB1leu group, and 592 mg in the TB8leu group. Leucine supplementation above the normal diet was 0.20 g/kg BW in the TB1leu group and 1.31 g/kg BW in the TB8leu group.

Skeletal muscle mass. At autopsy, the control group had more body weight; neither had it affected tumor weight. On day 20, food intake was significantly lower in the TB group than in the C group. The food intake between tumor-bearing groups did not differ (Table IB).

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Loss in the mEDL (fast twitch, more anaerobic) was relatively higher than in the mS (slow twitch, more aerobic). Skeletal muscle mass was higher in TB8Leu than in TB. All muscles from the TB8Leu group showed the same relative weight maintenance (Fig. 1), irrespective of muscle fiber type.

**Plasma amino acid concentrations.** At day 21 (Table III), plasma concentrations of total amino acids (AA), total essential amino acids (EAA) and total non-essential amino acids (NEAA) in TB were all higher than they were in control mice. In tumor-bearing mice, concentrations of all amino acids except methionine were higher, the greatest increases being in taurine (360%), lysine (204%), arginine (200%) and alanine (195%).

In the high-leucine group (TB8Leu), leucine supplementation significantly increased plasma leucine. In TB8Leu, total amino acids, total essential amino acids minus leucine and total non-essential amino acids were all lower than in TB. Of the individual amino acids, isoleucine, valine, lysine, alanine, asparagine, serine and glycine were all significantly lower in TB8Leu than in TB. Plasma AA levels in the TB1Leu group also tended to be lower than in the TB group, and serine and glycine levels were significantly lower. No effect on the ratio of tryptophan to large neutral amino acids (Table III) was observed in the tumor-bearing and leucine groups.

**Skeletal-muscle-breakdown parameters and MyoD.** Muscle (mEDL) mRNA levels of murf and atrogin, two skeletal-muscle-
specific ubiquitin ligases essential for protein breakdown (25), were higher in the cT group than in the c group. The tumors had no observable effects on myoD mRNA expression, a skeletal-muscle differentiation marker (26). Leucine supplementation did not affect any markers of muscle catabolism in mEdL. Plasma 3-methylhistidine concentrations were higher in the cT group than they were in the c group (Table IV).

Discussion

Tumor inoculation reduced carcass weight and the muscle masses of mG, mTA, mEdL and mS. While the carcass weights of the mice were not affected by leucine supplementation, high-leucine (cT8Leu) supplementation reduced the weight loss of both mG and mTA, suggesting that leucine has a specific effect on skeletal muscle.

An explanation for the reduction in muscle mass loss might be found in a reduction of protein breakdown. In the mEdL of the cT mice, the mRNA expression of the muscle-specific ubiquitin ligases atrogin-1 and murf, which both play an essential role in skeletal muscle protein breakdown, were up-regulated. We conclude that although leucine supplementation dose-dependently reduced the loss of skeletal muscle mass in the cT mice, this muscle mass gain was not accompanied by changes in expression of either ligase at day 21.

Effect of the tumor. The tumor induced muscle loss with a relative higher muscle loss in the mEdL (mainly fast more anaerobic type 2 fibers) when compared to the mS (mainly slow more aerobic type 1 fibers). This is in accordance with reports which show that during cancer cachexia oxidative muscle is broken down to a lesser extent (27).

In the cT cachectic mice, an increase in plasma amino acids (all, except methionine) was observed when compared to control mice. The increase in the concentration of total plasma amino acids in cT mice may have been due either i) to differences in food intake, or ii) to metabolic differences such as enhanced protein breakdown, increased transamination and conversion, or reduced plasma clearance (due to reduced protein synthesis or organ dysfunction) (28). However, the first possibility, an increased food intake, is unlikely, as food intake was lower in cT mice, and also relatively uniform, in all cT mice.

The fact that the concentration of methionine did not change in the presence of a tumor may be due to the rate-limiting effect of methionine for whole-body protein synthesis, as was previously suggested in AIDS patients (29). On the other hand, plasma methionine levels are probably kept in very narrow ranges in the body, a hypothesis that is supported by our finding that methionine concentrations did not correlate with muscle mass (data not shown). However, the exact reason still needs to be established.

The increased expression of murf and atrogin in the cT mice indicate that, at least in the mEdL, markers of protein breakdown are up-regulated in tumor-bearing mice. Another muscle protein breakdown marker is 3-methylhistidine, which, in urine, is usually used as a marker for skeletal muscle protein breakdown (30,31). Its plasma concentration has also been used as skeletal muscle protein breakdown marker in mice (32). Since the specificity of 3-methylhistidine concentrations for skeletal muscle breakdown has been challenged, because other organs might also contribute to its plasma and therefore urine levels (33,34), this parameter should be reviewed as an indicator in context with the other parameters.

The increase in plasma 3-methylhistidine concentrations in cachectic cT mice relative to c mice, and the correlation to muscle mass of all four muscles [R = -0.509 (mEdL), -0.583 (mS); -0.584 (mG); -0.541 (mTA); all p<0.001], suggests that muscle protein breakdown increased in the cT mice. In this context, it should be noted that skeletal muscle contains high amounts of taurine, and that, when skeletal muscle decreases in volume, taurine is expelled from the intracellular and interstitial spaces (35), leading to increased plasma taurine levels. In the C26 model, the taurine level did indeed increase dramatically in the cachectic mice, and was strongly

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<th>Table IV. Markers for proteolysis from plasma or mEdL.</th>
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<td>Fold induction (units)</td>
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C, mice receiving control diet (AIN93M); cT, tumor-bearing mice receiving control diet; cT1Leu, tumor-bearing mice receiving control diet supplemented with 1 g/kg leucine; cT8Leu, tumor-bearing mice receiving control diet supplemented with 8 g/kg leucine. Messenger RNA levels represent the fold induction of the different groups relative to C. For total RNA quantification, mRNA of GAPDH was used. Data are the means ± SEM: ‘a significandy different from TB (p<0.05).
negatively correlated with muscle mass \( R = -0.780 \) (mEDL), 
-0.607 (mS), -0.757 (mG); -0.729 (mTA); all \( p<0.001 \).

The presence of a tumor had no effect on MyoD mRNA expression in mEDL. Because MyoD is one of the factors that controls cell cycle propagation and the induction of differentiation of skeletal muscle cells (26,36), it plays a role in the growth and recovery of skeletal muscle. Our data therefore suggest that no significant role in the cachectic process was played by mRNA levels of the skeletal muscle differentiation marker (of anabolism) MyoD. This contrasts with the increase in mRNA levels of atrogin-1 and murf, both muscle-specific ubiquitin protein ligases involved in proteolysis (25), which was greater in the mEDL muscles of TB mice than it was in C mice.

**Effect of leucine supplementation in the tumor-bearing mice.** Our data on the effect of leucine on skeletal muscle mass support the findings of studies in healthy human volunteers which show that leucine can have a net anabolic effect on skeletal-muscle protein synthesis in young (37) and elderly men (38-40). It also supports the finding in diabetic and somatostatin-treated fasted rats that leucine stimulates signaling pathways, leading to protein translation through insulin-dependent and insulin-independent pathways (14,15). In those cases, the rats received 1.35 g leucine per kg BW by oral gavage, which is slightly higher than the highest leucine dose used in our own experiment (1.31 g/kg BW supplemented on top of the normal diet). These reported leucine-induced increases in protein synthesis are in accordance with Anthony et al (14,15) and other studies (reviewed in refs. 41-43). Thus, although muscle protein synthesis was not measured directly, our data suggest that leucine can, at least partly, compensate for the loss of anabolic effects of insulin on skeletal muscle during cancer cachexia. Because the addition of leucine did not change the parameters measured for catabolism, we hypothesize that the leucine-induced increase in muscle mass in this model was due mainly to an increased anabolism. Addition of 8 g leucine to 1 kg of food seemed to maintain muscle mass, in all different muscles relatively to a similar extent irrespective of the fiber type of the muscle, while muscle loss relatively appeared to be more pronounced in the muscles with more type II fibers.

Plasma leucine concentrations were only significantly increased in the TB8Leu group. In contrast, total plasma amino acids in TB8Leu were less than in TB. Thus, when leucine is provided in sufficient amounts, there seems to be a relationship between leucine supplementation and the reduction we measured in hyperaminoacidemia. This plasma amino acid decrease after leucine supplementation was dominated by the total non-essential amino acids, of which alanine decreased most. Because alanine is a product of intracellular transamination of free amino acids, which occurs when there is an increase in intracellular free amino acids, during proteolysis, for example (44), the increase in plasma amino acids, particularly alanine, might reflect an increase in the transamination of amino acids released from the muscle during protein breakdown.

Leucine supplementation had no effect on either the ubiquitin ligases, 3-methylhistidine or on taurine levels. Taken together, our findings, suggest that these parameters of protein breakdown are not affected by leucine in this model.

Although food intake was lower in the TB mice, it was not affected by leucine supplementation, which means that our results cannot be explained by differences in food intake. This contrasts with clinical data indicating that BCAA supplementation might increase food intake in cancer patients, malnourished patients, and patients with liver cirrhosis (45-49) (reviewed in ref. 50). One of the working mechanisms suggested for this orexigenic effect is that leucine reduces the tryptophan to large neutral amino acid ratio (46). The fact that supplementation with leucine did not change this ratio in the C26 mouse model seems consistent with leucine's lack of orexigenic effect in this model.

Leucine supplementation had no observable effects on the mRNA levels of either ubiquitin ligase. Together with the finding that leucine supplementation can increase skeletal muscle mass in cancer cachetic mice, the lack of effects of leucine supplementation on these ligases, and on plasma taurine and 3-methylhistidine concentrations, all suggest that the main effect of leucine on skeletal muscle protein balance is on the protein-synthesis side, not the protein-breakdown side. In summary, high leucine supplementation may contribute to a return to homeostasis.

While the net effect of leucine on the muscle masses was small, it was statistically significant. Because of the magnitude of the effect, it is doubtful whether supplementation of leucine alone can be considered clinically relevant in terms of the preservation of muscle mass in cancer. A multi-targeted approach can therefore be taken by combining leucine with other nutritional components. In a milder C26 model for cachexia without anorexia, the nutritional combination of leucine, high protein, fish oil and specific oligosaccharides (20,21,51-55) significantly reduced the loss of carcass, muscle and fat mass. Moreover, this specific combination also improved muscle performance, daily activity and immune function (Th1 response) (20,21). These results further strengthen the evidence that a balanced combination of ingredients might achieve multiple effects on the complex conditions of cachexia.

In conclusion, reductions in skeletal muscle mass and carcass weight in cachectic and anorectic mice were associated with higher skeletal muscle-protein breakdown in the C26 tumor-bearing mice model. This increased skeletal-muscle protein breakdown involved the ubiquitin ligases murf and atrogin-1. Leucine supplementation dose-dependently inhibited net muscle wasting, but had no effect on expression of the ligases or on body weight. The positive effect of leucine on the muscle was reflected in a reduction in the tumor-induced increase in plasma amino acid levels, suggesting a step forwards to return to homeostasis. For a clinically relevant effect, it might be more effective to combine leucine with other specific nutrients in a multi-target nutritional approach, and thereby, to target both anabolism and catabolism.

**References**


