Physiological characterization of a mouse model of cachexia in colorectal liver metastases

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ABSTRACT

Loss of skeletal muscle mass and function (cachexia) is severe in patients with colorectal liver metastases because of the large increase in resting energy expenditure but remains understudied due to a lack of suitable preclinical models. Our aim was to characterize a novel preclinical model of cachexia in colorectal liver metastases. We tested the hypothesis that mice with colorectal liver metastases would exhibit cachexia, as evidenced by a reduction in liver-free body mass, muscle mass and physiological impairment. Twelve week old male CBA mice received an intrasplenic injection of Ringer’s solution (sham) or murine colorectal cancer cells (MoCR) to induce colorectal liver metastases. At end-point (20-29 days), the livers of MoCR mice were infiltrated completely with metastases and MoCR mice had reduced liver-free body mass, muscle mass and epididymal fat mass compared with sham controls ($P<0.03$). MoCR mice exhibited impaired rotarod performance and grip strength ($P<0.03$). Histochemical analyses of tibialis anterior muscles from MoCR mice revealed muscle fiber atrophy and reduced oxidative enzyme activity ($P<0.001$). Adipose tissue remodeling was evident in MoCR mice, with reduced adipocyte diameter and greater infiltration of non-adipocyte tissue ($P<0.05$). These findings reveal the MoCR mouse model exhibits significant cachexia and is a suitable preclinical model of cachexia in colorectal liver metastases. This model should be utilized for identifying effective treatments for cachexia to improve quality of life and reduce mortality in patients with colorectal liver metastases.

KEYWORDS: Muscle wasting, cancer cachexia, muscle weakness, colorectal liver metastases
INTRODUCTION

Cancer cachexia is defined as a multifactorial syndrome defined by an ongoing loss of skeletal muscle mass (with or without loss of fat mass) that cannot be fully reversed by conventional nutritional support leading to progressive functional impairment (8). Cachexia occurs in up to 80% of patients with advanced cancers of the gastrointestinal tract, colon, lung, breast, sarcoma and prostate (33). It is also present early in the progression of gastrointestinal, pancreatic and lung cancers (33). Cachexia reduces mobility and functional independence, and causes severe fatigue, which together reduce overall quality of life (26). Cachexia can also increase the risk of post-operative complications, impair the response to anti-neoplastic treatments, and result in the eventual failure of respiratory and cardiac muscle function that causes 20-30% of all cancer-related deaths (33). Unfortunately, there is no FDA-approved treatment for cancer cachexia and this is due, at least in part, to a lack of suitable preclinical models that closely mimic the human condition for maximizing translational outcomes (24).

Colorectal cancer is the third most common cancer worldwide and is the fourth-most common cause of cancer-related death (9). The most frequent complication of colorectal cancer is the development of liver metastases, which occurs in 70% of cases and is the main cause of death in colorectal cancer patients (30). Only a small minority (~10%) of patients with colorectal liver metastases are considered candidates for resection which may offer a possible cure (30). For the remaining ~90% of patients, liver metastases are unresectable and chemotherapy is the main alternative. Cachexia is particularly prevalent and severe in patients with colorectal liver metastases due to the very high energy demands of the liver. In the healthy state, liver metabolism represents ~20% of whole body resting energy expenditure (REE) (7). However, REE increases in proportion to liver size and the large increase in liver mass due to metastases results in a greatly elevated REE (19). A prospective study of patients with colorectal liver metastases found that a 1 kg increase in liver mass (including metastases)
resulted in a 343 kcal increase in REE (19). The study also showed an accelerated loss of skeletal muscle and adipose tissue mass and an accelerated gain of liver mass in patients with colorectal liver metastases (19).

Treatments are needed urgently to counteract cachexia in patients with unresectable colorectal liver metastases, to improve their quality of life, enhance their response to chemotherapy, and to reduce mortality. Potential treatments need to be tested first in preclinical models that closely mimic the human condition but despite the availability of well-characterized preclinical models of non-metastatic colorectal cancer, such as the colon-26 (C-26) tumor-bearing mouse (24), no preclinical model of cachexia in colorectal liver metastases has been described. It is imperative that a model of cachexia in metastatic colorectal cancer is identified as the etiology and response to potential treatments is likely to be different to that in models of non-metastatic colorectal cancer. The aim of this study was to characterize the cachexia in our well-described mouse model of colorectal liver metastases (17, 18, 27). We tested the hypothesis that mice with colorectal liver metastases would exhibit cachexia, with reductions in liver-free body mass and muscle mass and impairments in whole body function, and therefore represent the first validated preclinical model of cachexia in colorectal liver metastases.

MATERIALS AND METHODS

**Experimental animals.** All experiments were approved by the Animal Ethics Committee of The University of Melbourne and conducted in accordance with the Australian code of practice for the care and use of animals for scientific purposes as stipulated by the National Health and Medical Research Council (Australia). Twelve week old male CBA mice (Laboratory Animal Services, The University of Adelaide, South Australia) were allocated randomly into one of two experimental groups: a sham control group (Sham, \( n = 12 \)) or a colorectal liver metastases group (MoCR, \( n = 12 \)). All mice were housed in the Biological
Research Facility at The University of Melbourne under a 12:12-hour light-dark cycle. Water was available ad libitum and both water and standard laboratory chow was provided, changed and monitored daily. The amount of food consumed per mouse per day was determined and expressed as cumulative food intake and the amount of water consumed per mouse per day was determined and expressed as cumulative water intake.

**Mouse model of colorectal liver metastases.** The mouse model of colorectal liver metastases was that described by Kuruppu et al. (17). A dimethyl-hydrazine-induced primary colon carcinoma was maintained by in vivo serial passages in the flanks of 10-12 week old male CBA mice. Tumors were removed from passage mice and used to make a tumor cell suspension (1 × 10^6 cells.ml⁻¹ in Ringers solution with 0.1% glucose). Three cohorts (n = 4/cohort for a total of n = 12 MoCR mice) of 12 week old mice were injected with MoCR cells that had been passaged once (cohort 1), twice (cohort 2) or four times (cohort 3). For tumor induction, mice were anesthetized with an intraperitoneal (i.p.) injection of a mixture of ketamine (100 mg.kg⁻¹ body mass) and xylazine (10 mg.kg⁻¹; VM Supplies, Chelsea Heights, Victoria, Australia), such that they were unresponsive to tactile stimuli. Preoperative carprofen (5 mg.kg⁻¹; Lyppard Australia, Keysborough, Victoria, Australia) was injected subcutaneously in the nape for pain relief. Mice were shaved on their side and the spleen exteriorized through a subcostal incision. Two hemostatic clips were applied adjacent to each other across the center of the spleen. Tumor cell suspension (0.05 ml) was slowly injected into the lateral portion of the spleen using a 25-gauge needle over a period of 1 min. The needle was retracted and even pressure was applied to the spleen for 2 min. A hemostatic clip was then applied across the splenic vessels supplying the section of spleen injected, and a portable cautery used to cauterize the splenic vessel following which a partial splenectomy was performed. The muscle and skin were sutured and mice were given a subcutaneous injection of atipamezole (Antisedan; 1 mg.kg⁻¹; VM Supplies) to partially reverse the effects of xylazine and promote more rapid recovery from sedation. Mice recovered on a heated pad
until fully conscious. This model has been characterized previously and results in metastases exclusively confined to the liver (17). Liver angiogenesis is established by day 10, followed by an exponential growth of tumors between days 10-16, and a plateau from day 19-22. Sham mice had a 0.05 ml injection of Ringers solution with 0.1% glucose and a partial splenectomy.

**Criteria for humane end point.** End point analyses were conducted if any of the following criteria were met: a decrease of >20% weight loss from the start of the experiment; a body condition score (BCS) of <2 as described previously (10); infection of the wound; or changes in respiration, vocalization and mobility.

**Grip strength and rotarod test.** Whole body strength and whole body mobility and coordination were assessed one day before end point analyses by means of a grip strength meter (Columbus Instruments, Columbus, OH) and rotarod performance test (Rotamex-5, Columbus Instruments) as described in detail previously (24). Grip strength was assessed five times within 2 min and the average grip strength was normalized to body mass. Rotarod performance was assessed three times with 15 min between tests and average latency-to-fall was calculated over the three trials.

**Dissections.** On the day of end point analyses, mice were anesthetized with an injection of 0.9% HEPES-buffered sodium pentobarbitone (Nembutal; 120 mg.kg⁻¹; Sigm-Aldrich, Castle Hill, NSW, Australia) via i.p. injection. Supplemental injections of unbuffered Nembutal (60 mg.kg⁻¹) were administered to maintain unresponsiveness to tactile stimuli. The tibialis anterior (TA), extensor digitorum longus (EDL), soleus, plantaris, gastrocnemius and quadriceps muscles, as well as the epididymal fat, heart, spleen and kidneys, were carefully excised, blotted on filter paper and weighed on an analytical balance. TA muscles were mounted in embedding medium and frozen in thawing isopentane for later histochemical and biochemical analyses. The TA muscle was chosen because: i) it is of sufficient mass and cross-sectional area for conducting these analyses; ii) we have found that it is susceptible to atrophy and functional impairment in the C-26 (24) and LLC tumor-bearing models (23); and
iii) we have previously investigated histochemical and biochemical properties of this muscle in the C-26 (24) and LLC tumor-bearing models (23). Although it would be of interest to also examine histochemical and biochemical properties in muscles predominantly composed of type I fibers, such as in the soleus muscle, the small mass of the soleus (5-6 mg) precludes its use in these analyses. Mice were killed as a consequence of heart excision while still anesthetized deeply.

Skeletal muscle histology. Serial sections (5 µm) were cut transversely through the TA muscle using a refrigerated (-20ºC) cryostat (CTI Cryostat; IEC, Needham Heights, MA). Sections were stained (or reacted) with: hematoxylin and eosin (H&E) to determine general muscle architecture; laminin (#L9393, Sigma-Aldrich) for determination of mean myofiber cross-sectional area (CSA); succinate dehydrogenase (SDH) to determine activity of oxidative enzymes; and N2.261 (developed by Dr. Helen M. Blau, obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by The University of Iowa, Department of Biology, Iowa City, IA, USA) to assess the percentage of myosin IIa isoforms (25). We have shown previously that mouse TA muscle contains a virtual absence of type I fibers (22) and so all non-N2.261 reacting fibers were assumed to represent type IIx/b fibers. Optical density (o.d.) of SDH was determined after 6 min of reactivity for all samples and sections were captured in full color using bright field light microscopy and analyzed, as described previously (25). Digital images were obtained using an upright microscope with camera (Axio Imager D1, Carl Zeiss, Wrek, Göttingen, Germany), controlled and quantified by AxioVision AC software (AxioVision AC Rel. 4.7.1, Carl Zeiss).

Fat histology. Epididymal fat was immersed in Bouin’s solution overnight and then transferred to 70% ethanol. Tissues were fixed and embedded in paraffin wax in a random orientation and 10 µm sections were cut using a cryostat (CTI Cryostat). Sections were stained with hematoxylin and eosin (H&E), imaged using an upright microscope and camera.
(Axio Imager F1) and quantified by AxioVision AC software. For each sample, diameter was determined in 251 ± 35 adipocytes (obtained from three independent sections of tissue).

**Real-Time RT-PCR analyses.** Total RNA was extracted from 10-20 mg of TA muscle using a commercially available kit according to the manufacturer’s instructions (PureLink RNA Mini Kit, Invitrogen). RNA concentration was determined spectrophotometrically at 260 nm, and the samples stored at -80°C. RNA was transcribed into cDNA using the Invitrogen SuperScript™ VILO cDNA Synthesis Kit, and the resulting cDNA stored at -20°C for subsequent analysis. Real-Time RT-PCR was carried out with the Bio-Rad CFX384 Touch Real-Time PCR Detection System (Bio-Rad, Hercules, CA) using the ssoAdvanced SYBR Green Supermix (Bio-Rad). Measurements included a no template control as well as an RT negative control. Primer sequences for MuRF-1, atrogin-1, IL-6 and TNF-α were as detailed previously (22, 24). The content of single-stranded DNA (ssDNA) in each sample was determined using the Quanti-iT OliGreen ssDNA Assay Kit (Molecular Probes, Eugene, OR), as described previously (25). Gene expression was quantified by normalizing the logarithmic cycle threshold (CT) value (2^{–CT}) to the cDNA content of each sample to obtain the expression 2^{–CT}/cDNA content (ng.ml⁻¹).

**Western blot analyses.** Western blotting was performed as described previously (16, 25). Membranes were incubated overnight at 4°C with the following antibodies (all 1:1,000 in blocking buffer): p-Akt (Ser473; #8271, Cell Signaling, Danvers, MA, USA); Akt (#9272, Cell Signaling); p-p70S6K (Thr389; #9205, Cell Signaling) and p70S6K (#9202, Cell Signaling) as detailed elsewhere (16, 25). The signal was imaged using ChemiDoc XRS machine (Bio-Rad) and blots were quantified using Image Lab software (Bio-Rad). A total protein stain (BLOT-FastStain™, G-Biosciences, St Louis, MO, USA) was performed by incubating membranes in Fixer for 3 min at RT, and then in Developer for 1 min at RT followed by 30 min at 4°C. The signal was imaged using ChemiDoc XRS machine and blots were quantified using Image Lab software.
**Statistical analyses.** All values are expressed as mean ± SE, unless stated otherwise. Groups were compared using a Student’s t-test, a one-way ANOVA or a two-way ANOVA, where appropriate. Fiber type proportions are presented as 95% confidence intervals of the mean. Differences were considered significant when no overlap existed between the 95% confidence intervals (31). Bonferroni’s post hoc test was used to determine significant differences between individual groups. Correlations were determined by least squares linear regression. The level of significance was set at $P < 0.05$ for all comparisons.

**RESULTS**

**Body condition score, food intake, body mass and liver mass in MoCR cohorts.** The body condition score (BCS) assessed the general health and well being of the mice and was used as a criterion for humane end point. The BCS of MoCR cohort 1 (mice injected with cells that been passaged once, P1) did not start declining until day 28 and end point analyses were performed on these mice on day 29 (Fig. 1A). In MoCR cohort 2 (mice injected with cells that had been passaged twice, P2), BCS started declining on day 24 and end point analyses were performed on day 26 (Fig. 1A). In MoCR cohort 3 (mice injected with cells that had been passaged four times, P4), BCS started declining on day 17 and end point analyses were performed on day 20 (Fig. 1A). There was a main effect for the BCS of cohort 3 to be lower than cohort 1 and cohort 2, and for the BCS of cohort 2 to be lower than cohort 1 ($P < 0.01$, Fig. 1A). The decline in BCS in cohorts 2 and 3 were associated with a plateau in cumulative food intake (Fig. 1B) and relative body mass (Fig. 1C). There was a main effect for food intake of cohort 3 to be lower than cohort 1 and cohort 2, and for food intake of cohort 2 to be lower than cohort 1 ($P < 0.01$, Fig. 1B). No significant difference between cohorts was found for body mass (Fig. 1C). Despite end point analyses being performed on different days, livers from the three cohorts were ~95% infiltrated with metastases (Fig. 1D), and there was no significant difference between cohorts in liver mass ($P < 0.84$, Fig. 1E),
indicating that the three MoCR cohorts had a similar extent of metastases. Liver mass in each of the MoCR cohorts was significantly higher than in sham controls ($P<0.01$, Fig. 1E). There were also no significant differences between MoCR cohorts in muscle mass ($P<0.98$, Fig. 1F). These findings confirm the appropriateness of using BCS in the criteria for humane end point. Because of the similarity in metastases infiltration, liver mass and muscle mass, data from the three cohorts were combined for comparison with sham controls which also comprised three cohorts that had their end point matched to the MoCR cohorts (cohort 1 end point, day 29 ($n=4$); cohort 2 end point, day 26 ($n=4$); cohort 3 end point (day 20).

**Body mass, body condition score, food and water intake, liver mass and liver-free body mass in MoCR mice.** Due to the invasive nature of the surgery, relative body mass of sham control mice decreased for the first 5 days after surgery and increased progressively thereafter (Fig. 2A). A similar initial decrease after surgery was seen in the MoCR mice but despite having an increase in liver mass due to metastases, MoCR mice had lower relative body mass than sham controls from day 10 (Fig. 2A). Sham mice maintained a healthy BCS of 3 over the course of the experiment (data not shown). MoCR mice ate and drank more than sham controls ($P<0.04$, Fig. 2B, C). At end point, MoCR mice had a ~6.5-fold higher liver mass than controls due to the infiltration of metastases (Sham, 1302 ± 59, $n=12$; MoCR, 8084 ± 852 mg, $n=9$; $P<0.001$). The increased liver mass was maintained when normalized for initial body mass ($P<0.001$, Fig. 2D). Calculation of liver-free body mass revealed that MoCR mice had a 24% reduction in mass compared with sham controls ($P<0.001$, Fig. 2E).

**Skeletal muscle and fat mass in MoCR mice.** MoCR mice had reduced mass of the soleus (Sham, 6.1 ± 0.4; MoCR, 4.8 ± 0.3 mg, $P<0.03$), EDL (Sham, 12.1 ± 0.5; MoCR, 8.6 ± 0.6 mg, $P<0.001$), TA (Sham, 44.1 ± 1.1; MoCR, 33.3 ± 1.2 mg, $P<0.001$), plantaris (Sham, 14.7 ± 0.4; MoCR, 9.1 ± 0.8 mg, $P<0.001$), gastrocnemius (Sham, 117.7 ± 3.1; MoCR, 91.8 ± 2.6 mg, $P<0.001$) and quadriceps muscles (Sham, 194.8 ± 5.1; MoCR, 140.8 ± 6.4 mg, $P<0.001$) compared with sham controls. When normalized for initial body mass, the lower
muscle masses in MoCR mice remained (Fig. 3A). MoCR mice had higher heart mass (Sham, 109.9 ± 3.5; MoCR, 136.4 ± 6.6 mg, \( P<0.01 \)) and spleen mass (Sham, 60.5 ± 3.1; MoCR, 96.2 ± 6.2 mg, \( P<0.001 \)) but lower white adipose tissue mass compared with sham controls (WAT, Sham, 810.1 ± 132.3; MoCR, 92.7 ± 37.3 mg, \( P<0.001 \)). There was no significant difference in kidney mass between groups (Sham, 420.8 ± 14.0; MoCR, 429.6 ± 18.9 mg).

When normalized for initial body mass, the higher spleen mass and lower WAT mass in the MoCR mice remained (Fig. 3B).

**Whole body strength and mobility in MoCR mice.** MoCR mice had a 21% lower average grip strength than sham controls (\( P<0.03 \), Fig. 4A). Latency-to-fall during the rotarod test was 82% lower in MoCR mice compared with sham controls (\( P<0.001 \), Fig. 4B). Impaired mobility has been correlated previously with weight loss in patients with cancer cachexia (13) and so the correlation between rotarod performance and the percentage change in liver-free body mass was investigated. When results for sham and MoCR groups were pooled (\( n=17 \)), there was a significant correlation (\( r=0.82, P<0.0001 \)) such that the greater the weight loss, the shorter the latency-to-fall (Fig. 4C).

**Muscle fiber size and oxidative enzyme activity in MoCR mice.** TA muscle cross sections were stained with H&E to assess general muscle fiber architecture, and reacted for myosin IIa (N2.261, green), laminin (red) and SDH activity (blue) to identify type IIa fibers, visualize all fibers, and indicate oxidative enzyme (SDH) activity, respectively (Fig. 5A). Type I fibers are very rare in mouse TA muscle so all non-N2.261 reacting fibers were assumed to represent type IIx/b fibers. H&E staining showed that MoCR mice had smaller muscle fibers but similar architecture and extent of non-muscle tissue as sham controls. There were no differences between groups in the proportion of type IIa and type IIx/b fibers (Fig. 5B) but MoCR mice had a 25% reduction in average fiber cross-sectional area (CSA, \( P<0.001 \)), which was due to atrophy of type IIa (-26%, \( P<0.001 \)) and type IIx/b fibers (-25%, \( P<0.001 \), Fig. 5C). Average fiber SDH reaction intensity was also lower in MoCR mice (-
19%, \( P<0.001 \)), due to lower SDH intensity in type IIa (-18%, \( P<0.001 \)) and type IIx/b fibers (-20%, \( P<0.001 \), Fig. 5D).

**Remodeling of adipose tissue in MoCR mice.** Examination of cross sections of epididymal fat stained for H&E showed substantial morphological changes in MoCR mice (Fig. 6A). Average adipocyte diameter was 49% smaller in MoCR mice (\( P<0.001 \), Fig. 6B) and a histogram revealed that this was due to a greater proportion of small adipocytes and a lower proportion of large adipocytes (\( P<0.05 \), Fig. 6C). Epididymal fat from MoCR mice also had a 5.6-fold larger area of non-adipocyte tissue (\( P<0.01 \), Fig. 6D).

**Pathways involved in protein degradation, inflammation and protein synthesis in MoCR mice.** To determine the mechanisms involved in the loss of muscle mass in MoCR mice, we assessed the expression of markers involved in muscle protein degradation (MuRF-1 and atrogin-1) and protein synthesis (Akt, p70 S6K) in TA muscles. Since inflammation plays an important role in the pathogenesis of cancer cachexia (2), the expression of inflammatory genes (IL-6 and TNF-\( \alpha \)) was also assessed. MuRF-1 and atrogin-1 mRNA expression was one-fold and 11-fold higher in MoCR mice compared with sham controls, respectively (\( P<0.03 \), Fig. 7A, B). IL-6 mRNA expression was two-fold higher in MoCR mice compared with controls (\( P<0.01 \), Fig. 7C), but there was no significant difference in TNF-\( \alpha \) mRNA expression between groups (Fig. 7D). To examine changes in the protein synthesis pathway, the expression of phosphorylated and total Akt and p70 S6K was assessed (Fig. 8A). There were no significant differences between groups in phosphorylated and total Akt (Fig. 8B-D) but MoCR mice had a 40% lower expression of total p70 S6K (\( P<0.04 \), Fig. 8F), and a tendency for lower phosphorylated p70 S6K (\( P<0.08 \), Fig. 8E). As a consequence, phosphorylated p70 S6K normalized to total p70 S6K was not different between groups (Fig. 8H).
DISCUSSION

Cachexia is particularly prevalent and severe in patients with colorectal liver metastases, but remains understudied because of the lack of suitable preclinical models. Preclinical models are essential for our understanding of the pathogenesis and relevance of potential therapies. Until now, no preclinical model of cachexia in colorectal liver metastases has been characterized. Because the etiology and response to potential treatments is likely to be very different between models of metastatic and non-metastatic colorectal cancer, it is essential that a model of cachexia in metastatic colorectal cancer is identified and validated. We report here for the first time a novel preclinical model of cachexia in colorectal liver metastases. In a mouse model of colorectal liver metastases (MoCR) where the intrasplenic injection of murine colorectal cancer cells results in significant liver metastases (17), mice exhibited reductions in body mass, muscle mass and fat mass as well as whole body physiological impairments. These changes are consistent with the diagnostic criteria for cachexia (8) and therefore confirm the suitability of this model for preclinical studies. Identification of this model represents a significant advance for the translation of preclinical findings and should be used to identify effective treatments for cachexia to improve the quality of life and survival of patients with colorectal liver metastases.

Reproducibility of liver metastases and cachexia in the MoCR model. Three cohorts of mice were injected with MoCR cells that had been passaged once (P1), twice (P2) or four times (P4). The greater the passage number of injected cells, the faster the deterioration in body condition score and plateau in food intake and body mass. Despite these differences, the infiltration of metastases was similar between cohorts (~95%). Liver mass and mass of various skeletal muscles was also similar between cohorts, demonstrating the reproducibility of liver metastases and cachexia in the MoCR model. They also show that injection of MoCR cells of differing passages can be used to produce varying speeds of disease progression or when examined at a single time point, can be used to examined different stages of cancer
cachexia (i.e. at day 20, P4 cells produce severe cachexia, P2 cells produce cachexia and P1
cells produce mild cachexia).

**Weight loss in the MoCR model of colorectal liver metastases.** The main diagnostic
criteria for cachexia in humans is a >5% weight loss (8). Weight loss of >15% is associated
with impaired physiological function and death ensues when weight loss is ~30% (36). In the
present study, liver-free body mass was reduced by 24% in mice with colorectal liver
metastases compared with sham controls. In comparison, we have shown previously that
severely cachectic mice bearing non-metastatic subcutaneous colorectal tumors (C-26) lost
~22% tumor-free body mass (24). Anorexia often but not always accompanies cancer
cachexia and can lead to a reduction in nutrient intake of 300-500 kcal/day (32). We have
shown previously that severely cachectic mice bearing non-metastatic C-26 tumors have
anorexia (24) but in the current study, when results for the three MoCR cohorts of mice with
colorectal liver metastases were combined, anorexia was not observed and they actually ate
and drank more than controls. Previous studies in patients with colorectal liver metastases
have shown that despite cachectic patients exhibiting deterioration in depression and physical
symptoms, they do not eat less than non-cachectic patients (12). These findings reveal that
cachexia in mice with colorectal liver metastases is not due to anorexia. Since nutritional
supplementation and pharmacological modulation of appetite does not enhance lean muscle
mass, these findings are also consistent with the notion that anorexia is not the sole
contributor to cancer cachexia (36). The small increase in food intake with the loss of body
mass indicates that the MoCR mice may have increased energy expenditure. Although we
were unable to directly assess energy expenditure in the present study, a prospective study of
patients with colorectal liver metastases found that a 1 kg increase in liver mass (including
metastases) resulted in a 343 kcal increase in energy expenditure (19). Assuming a similar
relationship exists in mice, the 6.8 g increase in liver mass in the MoCR mice equates to a
2.32 kcal/day elevation in energy expenditure. We have shown previously that 15 week old
mice have an energy expenditure of ~14.5 kcal/day (24), and so an elevation of 2.32 kcal/day would equate to a 15% increase in energy expenditure. Together with the reductions in body mass and muscle fiber oxidative enzyme activity (SDH intensity), an increase in energy expenditure in the MoCR mice suggests energy insufficiency and this has recently been shown in a rat model of cancer cachexia (11). Future studies should confirm this by directly measuring energy expenditure.

Muscle wasting in the MoCR model of colorectal liver metastases. The magnitude of skeletal muscle loss varies considerably in patients with cancer cachexia. A retrospective study of patients with colorectal liver metastases reported an ~16% loss of estimated whole body muscle mass (~4.2 kg) measured at 10.7 to 1.2 months from death (19). In the present study, the mass of muscles of varying fiber types were all significantly (17-37%) smaller in MoCR mice compared with sham controls. This is in contrast to severely cachectic C-26 tumor-bearing mice that demonstrated only (19-21%) reductions in the mass of larger muscles and not in the smaller soleus and plantaris muscles (24). In addition to a loss of muscle mass, patients with cancer cachexia exhibit muscle fiber atrophy with one study reporting a 32% smaller muscle fiber size in cachectic patients with gastrointestinal cancer compared with healthy controls (37). Muscle fiber atrophy was also found in the MoCR mice, with a 25% decrease in fiber area consistent across the fast oxidative type IIa fibers and fast glycolytic type IIx/b fibers. Real-Time RT-PCR and western blotting analyses revealed that the muscle atrophy in MoCR mice involved increased expression of protein degradative pathways (MuRF-1 and atrogin-1), increased inflammatory markers (IL-6) and reduced expression of protein synthesis pathways (p70 S6K). Each of these changes has been reported in animal models of cachexia in non-metastatic cancer (6, 24, 38), and suggest that the loss of muscle mass in MoCR mice may be due to both decreased signalling of the pathways regulating protein synthesis and increased signalling of the pathways regulating protein degradation.
Severe fatigue affects 70-100% of patients with cancer cachexia and is one of the main factors contributing to reduced quality of life (4). Decreased activity of oxidative enzymes is associated with fatigue and has been reported in patients with cancer cachexia (29). MoCR mice had reduced oxidative enzyme activity as evidenced by a reduction in SDH activity in type IIa and type IIx/b fibers. MoCR mice therefore exhibit similar reductions in muscle mass, fiber size and oxidative enzyme activity to patients with cancer cachexia.

The MoCR model of colorectal liver metastases has physiological impairments. One of the most debilitating aspects of cancer cachexia is the impairment in whole body strength and mobility. Grip strength is reduced by more than 25% in patients with cancer cachexia (14), affecting their ability to perform everyday tasks such as rising from a chair or bed, perform home duties and maintain personal hygiene. The reduction in grip strength in cachectic patients has also been correlated strongly with postoperative complications (14). We have shown previously that both severely cachectic and mildly cachectic mice bearing non-metastatic C-26 tumors have a 22% reduction in grip strength compared with controls (24) and in the present study, MoCR mice had a 21% reduction in grip strength. Thus, the magnitude of impairment in grip strength in our non-metastatic C-26 and metastatic MoCR models is very similar to that in cachectic cancer patients and highlights the suitability of these preclinical models.

The impaired mobility in patients with cancer cachexia results in reduced levels of physical activity (5). MoCR mice exhibited a large 82% impairment in mobility as assessed by rotarod performance. This is consistent with the reduction in mobility we have reported previously in severely cachectic C-26 tumor-bearing mice (24), and the consistency between models demonstrates that the impaired mobility in the MoCR mice was not simply due to the large tumor burden in the abdomen. The impaired mobility in patients with cancer cachexia has been correlated significantly with weight loss (13), and we also found a significant
correlation between weight loss and impaired mobility; highlighting the clinical relevance of
the MoCR model.

**Fat loss and remodeling in the MoCR model of colorectal liver metastases.** Cachexia
is usually accompanied by a loss of fat mass which often precedes the loss of muscle mass.
Similar to the loss in muscle mass, the most rapid loss of fat in patients with colorectal liver
metastases occurs within two months of death (19). In addition to the loss of fat mass, patients
with cancer cachexia experience adipose tissue remodeling characterized by the shrinkage of
adipocytes and an increase in interstitial spaces (21). Similar to humans, MoCR mice had a
loss of fat mass, reduced adipocyte size and an increased proportion of non-adipocyte tissue.
The same characteristics are found in severely cachectic C-26 tumor-bearing mice (34).

An interesting observation in the present study was the increase in spleen and heart
mass in the MoCR mice. The increase in spleen mass is consistent with patients with
colorectal liver metastases (19) and the non-metastatic C-26 model of cancer cachexia (35).
The cardiac hypertrophy is in contrast to the reduced absolute heart mass reported previously
in C-26 tumor-bearing mice (24, 35). However, these same studies found that C-26 tumor-
bearing mice had greater heart mass normalized to tumor-free body mass, which was
attributed to edema (24, 35). Chronic liver disease is commonly associated with portal
hypertension and the increase in portal pressure when the splenic vein drains into the portal
system can lead to an increase in splenic venous pressure (15). Portal hypertension is a well
documented cause of spleen enlargement (20) and cardiac hypertrophy (28) and is the likely
cause of the increased heart and spleen mass in MoCR mice.

**Perspectives and Significance**

Despite cancer cachexia being a devastating condition affecting more than 80% of
cancer patients and causing the death of more than 20-30% of all cancer patients, there is
currently no treatment. A contributing reason for the lack of progress has been a lack of
suitable preclinical models that closely mimic the human condition necessary for maximizing translational outcomes. Cachexia is particularly severe in patients with colorectal liver metastases but no preclinical model of cachexia in colorectal liver metastases has been identified. We have identified a novel, reproducible mouse model of colorectal liver metastases with cachexia that represents a suitable preclinical model. Several models of cachexia in non-metastatic colorectal cancer have been characterized but this is the first model of cachexia in metastatic colorectal cancer. Since liver metastases affects 70% of patients with colorectal cancer, this model represents a significant advance that will enhance the translation of preclinical findings. This model will increase the chances of identifying an effective treatment for cancer cachexia to improve the quality of life, enhance the response to antineoplastic treatments, and reduce mortality in patients with colorectal liver metastases.
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DEDICATIONS

This manuscript is dedicated to one of the authors, Ms. Cathy Malcontenti-Wilson, who passed away on February 14, 2013.

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COMPETING INTERESTS

The authors declare that they have no competing interests.
REFERENCES


metabolism, microcirculation, and strength of skeletal muscles in cancer-related cachexia.


FIGURE LEGENDS

Fig. 1. Comparison of body condition score (BCS), food intake, body mass, liver mass and muscle mass between three cohorts of CBA mice injected intrasplenically with colorectal cancer cells (MoCR) that had been passaged once (cohort 1, P1), twice (cohort 2, P2) or four times (cohort 3, P4). BCS (A), food intake (B) and body mass (C) were recorded daily. Arrows indicate the end points for each cohort. At end point, the liver was surgically excised (D) and weighed (E), and various hindlimb skeletal muscles were excised and weighed (F).

EDL, extensor digitorum longus; Plant, plantaris; TA, tibialis anterior; Gastroc, gastrocnemius; Quad, quadriceps. Data are means ± SE; n = 4. *P < 0.01 vs. sham; aP < 0.01 cohort 1 (P1) vs cohort 2 (P2) main effect; bP < 0.01 cohort 1 (P1) vs cohort 3 (P4) main effect; cP < 0.01 cohort 2 (P2) vs cohort 3 (P4) main effect.

Fig. 2. Analysis of body mass, food and water intake, liver mass and liver-free body mass in CBA mice injected intrasplenically with Ringer’s solution alone (sham) or containing colorectal cancer cells (MoCR). Relative body mass (A), food intake (B) and water intake (C) were recorded daily. At end point, the liver was surgically excised and weighed, allowing for calculation of liver mass normalized for initial body mass (D) and liver-free body mass (E).

Data are means ± SE; n = 12 [combined data from three cohorts of sham mice (n = 4/cohort) and MoCR mice (n = 4/cohort)]. *P < 0.05 vs. sham; aP < 0.04 sham vs MoCR main effect.

Fig 3. Mass of selected muscles and organs in CBA mice injected intrasplenically with Ringer’s solution alone (sham) or containing colorectal cancer cells (MoCR). Mass normalized for initial body mass of selected hindlimb muscles (A) and of heart, kidneys, epididymal white adipose tissue (WAT) and spleen (B). EDL, extensor digitorum longus; Plant, plantaris; TA, tibialis anterior; Gastroc, gastrocnemius; Quad, quadriceps. Data are
means ± SE; n = 12 [combined data from three cohorts of sham mice (n = 4/cohort) and MoCR mice (n = 4/cohort)]. *P < 0.05 vs. sham.

Fig 4. Whole body grip strength and rotarod performance and relationship between rotarod performance and weight loss in CBA mice injected intrasplenically with Ringer’s solution alone (sham) or containing colorectal cancer cells (MoCR). Whole body strength was assessed using a grip strength meter and normalized for body mass (A), and whole body mobility was assessed via latency-to-fall during a rotarod test (B). The relationship between weight loss expressed as the percentage change in liver-free body mass from initial and latency-to-fall during a rotarod test was examined (C). Regression equation: y = 1.91x + 67.38, r = 0.82, P < 0.0001, n = 17. Data are means ± SE; n = 12 [combined data from three cohorts of sham mice (n = 4/cohort) and MoCR mice (n = 4/cohort)]. *P < 0.03 vs. sham.

Fig. 5. Fiber type proportions, fiber cross-sectional area and fiber oxidative enzyme activity of TA muscles from CBA mice injected intrasplenically with Ringer’s solution alone (sham) or containing colorectal cancer cells (MoCR). Representative images (20×) of muscle sections reacted for hematoxylin and eosin (H&E) to assess general muscle architecture (A). Representative images (20×) of muscle sections reacted for laminin (red), myosin IIa (N2.261, green) or SDH (blue), indicating individual fibers, type IIa fibers and oxidative enzyme activity, respectively (A). Merged images for laminin, myosin IIa and SDH are also shown. Quantification of laminin, N2.261 and SDH activity based on reaction intensity facilitated determination of the percentage of type IIa and type IIx/b fibers (non-N2.261 reacting fibers, B); the area of type IIa and type IIx/b fibers (C); and the SDH activity based on reaction intensity of type IIa and type IIx/b fibers (D). Data are means ± SE; n = 12 for sham [combined data from three cohorts of sham mice (n = 4/cohort)], 6 for MoCR
combined data from three cohorts of MoCR mice (n = 2/cohort). *P < 0.01 vs. sham. Scale 
bars = 100 µm.

Fig. 6. Architecture and adipocyte area of epididymal fat from CBA mice injected 
intrasplenically with Ringer’s solution alone (sham) or containing colorectal cancer cells 
(MoCR). Representative images (20×) of cross sections reacted for hematoxylin and eosin to 
assess general fat architecture (A). Average adipocyte diameter (B), distribution of adipocyte 
diameter (C), and the percentage of non-adipocyte area (D). Data are means ± SE; n = 6 for 
sham [combined data from three cohorts of sham mice (n = 2/cohort)], 7 for MoCR 
[combined data from three cohorts of MoCR mice (n = 2-3/cohort)]. *P < 0.05 vs. sham. 
Scale bars = 100 µm.

Figure 7. Gene expression of the ubiquitin ligases MuRF-1 and atrogin-1 and the 
inflammatory markers IL-6 and TNF-α in TA muscles from CBA mice injected 
intrasplenically with Ringer’s solution alone (sham) or containing colorectal cancer cells 
(MoCR). Real-Time RT-PCR analysis of MuRF-1 (A), atrogin-1 (B), IL-6 (C) and TNF-α 
(D). Data are SE; n = 10 for sham [combined data from three cohorts of sham mice (n = 3- 
4/cohort)], 8 for MoCR [combined data from three cohorts of sham mice (n = 2-3/cohort)]. *P 
< 0.03 vs. sham.

Figure 8. Expression of phosphorylated and total Akt and p70 S6K in TA muscles from CBA 
mice injected intrasplenically with Ringer’s solution alone (sham) or containing colorectal 
cancer cells (MoCR). Representative images (A) and group data for (B) phosphorylated Akt 
(Ser473) normalized to total protein (B), Akt normalized to total protein (C), phosphorylated 
Akt (Ser473) normalized to total Akt (D), phosphorylated p70 S6K (Thr389) normalized to total 
protein (E), p70 S6K normalized to total protein (F) and phosphorylated p70 S6K (Thr389)
normalized to total p70 S6K ($G$). Data are SE; $n = 7$ [combined data from three cohorts of
sham mice ($n = 2-3$/cohort) and three cohorts of MoCR mice ($n = 2-3$/cohort)]. #$P < 0.08$ vs.
sham, *$P < 0.04$ vs. sham.
Figure 3.
Figure 4.

(A) Average grip strength/body mass (kg.g⁻¹) for Sham and MoCR groups.

(B) Average latency-to-fall (sec) for Sham and MoCR groups.

(C) Scatter plot showing the correlation between weight loss (% change liver-free body mass) and latency-to-fall (s). The correlation coefficient (r) is 0.82, with a p-value < 0.0001.
Figure 6.

A) Histological images showing differences between Sham and MoCR groups.

B) Bar graph comparing adipocyte diameter (μm) between Sham and MoCR groups.

C) Bar graph showing the percentage of adipocytes across different diameter ranges for Sham and MoCR groups.

D) Bar graph showing the percentage of non-adipocyte area between Sham and MoCR groups.
Figure 7.

Panel A: MtRF-1 mRNA/CDNA content (2^(-ΔΔCt) × 10^(-13)).

Panel B: Atrogin-1 mRNA/CDNA content (2^(-ΔΔCt) × 10^(-13)).

Panel C: IL-6 mRNA/cDNA content (2^(-ΔΔCt) × 10^(-13)).

Panel D: TNIIF-α mRNA/cDNA content (2^(-ΔΔCt) × 10^(-13)).