Habitual Myofibrillar Protein Synthesis Is Normal in Patients with Upper GI Cancer Cachexia

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Abstract

Purpose: Skeletal muscle wasting and weight loss are characteristic features of cancer cachexia and contribute to impaired function, increased morbidity, and poor tolerance of chemotherapy. This study used a novel technique to measure habitual myofibrillar protein synthesis in patients with cancer compared with healthy controls.

Experimental design: An oral heavy water (87.5 g deuterium oxide) tracer was administered as a single dose. Serum samples were taken over the subsequent week followed by a quadriceps muscle biopsy. Deuterium enrichment was measured in body water, serum alanine, and alanine in the myofibrillar component of muscle using gas chromatography–pyrolysis–isotope ratio mass spectrometry and the protein synthesis rate calculated from the rate of tracer incorporation. Net change in muscle mass over the preceding 3 months was calculated from serial CT scans and allowed estimation of protein breakdown.

Results: Seven healthy volunteers, 6 weight-stable, and 7 weight-losing (≥5% weight loss) patients undergoing surgery for upper gastrointestinal cancer were recruited. Serial CT scans were available in 10 patients, who lost skeletal muscle mass progressively at a rate of 5.6%/100 days. Myofibrillar protein fractional synthetic rate was 0.058%, 0.061%, and 0.073%/hour in controls, weight-stable, and weight-losing patients, respectively. Weight-losing patients had higher synthetic rates than controls (P = 0.03).

Conclusion: Contrary to previous studies, there was no evidence of suppression of myofibrillar protein synthesis in patients with cancer cachexia. Our finding implies a small increase in muscle breakdown may account for muscle wasting. Clin Cancer Res; 21(7); 1734–40. ©2014 AACR.

Introduction

Loss of skeletal muscle mass in cancer is well described (1–3) and is a defining characteristic of the cachexia syndrome (4). Low muscle mass is associated with a variety of clinical consequences including prolonged hospitalization, reduced mobility, and increased chemotherapy toxicity (5). A key objective of supportive cancer care is to preserve or increase muscle mass and perhaps improve outcomes during cancer treatment. In normal day-to-day living, muscle mass is generally thought to be maintained by the balance between muscle protein synthesis and breakdown. An alternative process is the balance between muscle regeneration via activation of satellite cells and myonuclear loss via apoptosis (6).

A reduction in muscle mass can occur due to an increase in muscle protein breakdown, a decrease in synthesis, or a combination of the two and all variations have been described in animal models of cancer cachexia (7). However, there are many limitations when extrapolating such models to the human situation, including the more acute versus chronic timescales of tumor growth and development of muscle wasting (8). Because of the invasive nature of clinical protocols to date, there are few studies of skeletal muscle protein metabolism in human cancer cachexia and these have demonstrated either markedly reduced (9) or unchanged (2) fractional synthetic rate (FSR) when compared with controls. In a previous study of patients with upper gastrointestinal cancer cachexia, we have demonstrated that the transcriptome in human skeletal muscle demonstrates downregulation of approximately 1,700 genes (mostly connected with cell turnover) and upregulation of approximately 100 genes, none with known connections to protein degradation via the ubiquitin proteasome pathway (10). This led to the conclusion that reduced synthesis was the likely cause of muscle wasting in such patients.

Previous studies of skeletal muscle protein synthesis in humans have used an infusion protocol with a stable isotope labeled amino acid. Under controlled conditions in a clinical research facility, such protocols are ideal for estimating the short-term response (2–6 hours) to specific stimuli such as feeding or exercise, but are unsuitable for measuring skeletal muscle synthesis in individuals living in the community. An alternative method uses the oral administration of a deuterium tracer in the form of heavy water (H2O). With this method, endogenous or intrinsic labeling of dispensable amino acids occurs and the latter become incorporated into muscle protein. The predictable kinetics and long elimination half-time of body water allows a period

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Translational Relevance

Cachexia affects the majority of patients with cancer with advanced disease and its treatment represents a major unmet clinical need. Muscle wasting is a key feature of the cachexia syndrome. Although studied in animal models, there are few studies in patients and none measuring muscle protein synthesis in free-living subjects at home. Here, we have measured muscle protein synthesis in patients before undergoing surgery for upper gastrointestinal cancer. We show that muscle protein synthesis is not decreased and may even be marginally increased. Our findings support the concept that a small increase in protein breakdown or altered myonuclear regeneration/loss may be the main mechanism accounting for muscle wasting in patients with cancer. Our findings suggest that multimodal cachexia therapy should utilize anabolic strategies (as synthesis is not suppressed) and perhaps also target specific breakdown pathways/myonuclear regeneration to achieve maximum benefit.

Materials and Methods

Subjects/protocol

The protocol received approval by the Lothian Research Ethics Committee. Written informed consent was obtained from all participants. All procedures were in accordance with International Conference on Harmonization Guidelines on Good Clinical Practice and the Helsinki Declaration. Healthy volunteers were screened using a simple health questionnaire (18), patients planned for upper gastrointestinal cancer surgery were recruited at the Royal Infirmary Edinburgh (Edinburgh, United Kingdom). A medical history was recorded along with current height and weight and a self reported preillness weight from which total body water (TBW) was calculated from deuterium enrichment in the study. For the healthy volunteers, a quadriceps biopsy was taken under local anesthetic using a Bergstrom needle and subsequently snap-frozen in liquid nitrogen. Combined quadriceps and rectus abdominis biopsies were taken under general anesthesia at the start of surgery in the patients with cancer.

Laboratory methods

The procedure for preparation and analysis of samples was performed as previously described (17). A brief outline is described below.

Serum samples

One milliliter of serum was used for analysis in triplicate of 2H enrichment in body water by continuous flow IRMS following equilibration with H2 gas (19). One milliliter of each serum sample was deproteinsed by ultrafiltration before analysis of free amino acid enrichment. Plasma-free amino acids were purified by cation exchange and prepared for GC-P-IRMS analysis as ethoxy carbonyl ethyl esters (17). Basal deuterium abundance in protein-bound alanine from serum albumin was measured (20) and assumed to be equal to basal abundance in myofibrillar protein (21).

Processing of muscle biopsies

Rectus and quadriceps skeletal muscle biopsies were processed to isolate the myofibrillar component. Muscle biopsies of approximately 30 μg were homogenized on ice and centrifuged at 10,000 × g for 15 minutes. The supernatant underwent serial Triton X-100 washes and centrifugation with the myofibrillar pellet being retained. Rectus abdominis muscle was additionally processed for recovery of free intracellular amino acids. After homogenization and centrifugation, the initial supernatant was processed for separation of free intracellular amino acids by ultrafiltration. Myofibrillar protein isolates (~2 mg) were subjected to gas phase acid hydrolysis (6 mol/L HCl, 150°C for 4 hours) and the resulting amino acids were derivatized as ethoxy carbonyl ethyl esters before analysis of deuterium enrichment and the relative concentration of individual neutral amino acids by GC-P-IRMS (17).

GC-P-IRMS of amino acids

In basic outline, 4 μL aliquots of the volatile amino acid derivatives from protein hydrolysates and free amino acids were injected into the GC-P-IRMS with the injector at 250°C and the column oven at 50°C. After a 1-minute hold, amino acids were separated on a 60 m × 0.32 mm × 0.5 μm film DB-Wax column programmed to 150°C at 25°C/minute, then to 220°C at 2.5°C/minute and then at 7.5°C/minute to 250°C, where it was held for 1 minute. Through the action of a microfluidics Deans switch, neutral amino acids were diverted online into a 1,350°C capillary pyrolysis furnace to convert analytes to H2 gas. The H2 gas entered the IRMS ion source through an open/split separator. H2 was ionized and hydrogen isotopes were separated and their abundance measured by comparison with a reference gas.

Body composition

Deuterium dilution space was calculated from the intercept of the elimination plot divided by the dose, following the methodology as used in the doubly labeled water protocol (22). Total body water (TBW) was calculated from deuterium space assuming nonaqueous hydrogen exchange factor of
1.041. Fat-free mass was calculated from TBW by assuming FFM hydration was 73.2% (23). Skeletal muscle mass (SMM, kg) was estimated from FFM using the MRI-validated equations of Wang and colleagues (24), which describe the ratio of SMM:FFM with gender and age. A myoﬁbrillar protein content of 12.4% SMM was assumed (25).

CT measures of body composition

Cross-sectional measures of muscularity were taken from routine CT scans in patients in the preoperative period according to the previously described method (26). Muscle cross-sectional area was measured with SliceOmatic V4.3 software (Tomovision). To provide clinical context and to allow comparison with total body water measures of body composition, (Tomovision). To provide clinical context and to allow comparison with total body water measures of body composition, cross-sectional measurements were converted to whole body estimates of FFM using the formula of Mourtzakis and colleagues (26). Muscle mass values were adjusted for height and estimates of FFM using the formula of Mourtzakis and colleagues (26). Muscle mass values were adjusted for height and estimates of FFM using the formula of Mourtzakis and colleagues (26).

Skeletal muscle myoﬁbrillar protein FSR

When patients were categorized by weight loss, there was a trend in quadriceps myoﬁbrillar protein FSR across all three groups (healthy controls < weight stable patients with cancer < weight-losing patients with cancer; P = 0.022). Quadriceps FSR was higher in weight-losing patients with cancer (0.079%/hour) compared with healthy volunteers (0.058%/hour; Fig. 1; P = 0.03). Quadriceps myoﬁbrillar protein FSR was 0.058% per hour in healthy volunteers compared with 0.067% per hour in all patients with cancer (P = 0.079).

Comparison of quadriceps and rectus FSR within weight-loss categories revealed no difference between quadriceps and rectus in the weight-stable group (P = 0.34) but signiﬁcantly higher in quadriceps versus rectus in the weight-losing patients.
Myofibrillar Protein Synthesis in Cancer Cachexia

Figure 1.
Category scatterplot of quadriceps myofibrillar protein FSR (%/hour) for healthy controls, weight-stable patients, and weight-losing patients with cancer. Horizontal line marks median value. ¶, P = 0.021. Post hoc comparisons revealed no significant associations (Spearman ρ) between quadriceps FSR or rectus FSR with skeletal muscle index (P = 0.322; P = 0.537), mean Hounsfield units (P = 0.583; P = 0.625), or the presence of myosteatosis (P = 0.544; P = 0.856). Low muscularity was not associated with altered quadriceps FSR (P = 0.463), low muscularity quadriceps FSR = 0.063%/hour (range 0.043–0.082), and normal muscularity FSR = 0.067%/hour (range 0.058–0.074). However, rectus FSR was greater in low muscularity patients (FSR = 0.063%/hour, range 0.042–0.069) than normal muscularity patients (FSR = 0.046%/hour, range 0.036–0.061; P = 0.038, Mann–Whitney U test).

Baseline body composition and preoperative loss of skeletal muscle mass

CT scans were undertaken immediately before surgery for all 14 patients with cancer. A measure of skeletal muscle cross-sectional area at the level of the third lumbar vertebrae was available for each. Of these 14 patients, 9 were classified with low muscularity and 5 were classified as myosteatotic. Sequential scans were available in 10 patients with a mean interval time of 79 days. Summary baseline (i.e., before neoadjuvant chemotherapy) body composition measures are shown in Table 2. Over the subsequent 79 days, all but one patient lost muscle mass. Median muscle loss was 5.6%/100 days (Figure 2). There was no correlation between muscle loss and muscle FSR using either quadriceps or rectus measures (P = 0.531; P = 0.940).

Table 2. Baseline body composition measures derived from CT in all patients with cancer (n = 14)

<table>
<thead>
<tr>
<th>Body composition measures</th>
<th>Median</th>
<th>IQR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Skeletal muscle mass index, cm²/m²</td>
<td>41.7</td>
<td>39.3–47.7</td>
</tr>
<tr>
<td>Mean HU</td>
<td>36.3</td>
<td>32.3–38.8</td>
</tr>
<tr>
<td>CT-derived fat-free mass, kg</td>
<td>44.8</td>
<td>39.8–51.2</td>
</tr>
<tr>
<td>CT-derived fat mass</td>
<td>24.6</td>
<td>19.6–27.7</td>
</tr>
<tr>
<td>Fat-free mass (kg; deuterium dilution)</td>
<td>46.3</td>
<td>41.9–49.8</td>
</tr>
<tr>
<td>Fat mass (kg; deuterium dilution)</td>
<td>28.6</td>
<td>24.9–31.4</td>
</tr>
</tbody>
</table>

Myofibrillar protein synthesis and breakdown

Skeletal muscle FBR was not different between weight-stable (n = 3, FBR = 0.056%/hour, range –0.049–to–0.067) and weight-losing patients with cancer (n = 7, FBR = 0.061, range –0.037–to–0.075; P = 0.833). Skeletal muscle mass estimated from deuterium dilution in only those patients with cancer who underwent serial CT scans is given in Table 3. Loss of muscle mass, as revealed by CT scans, enabled FBR data to be calculated in the patients in comparison with the control subjects, who were assumed to have stable muscle mass (thus, FSR = FBR). These early-stage patients with cancer showed similar muscle mass to controls and similar level of relative (FSR) and absolute myofibrillar protein synthesis (Table 3). In the patients with cancer, myofibrillar protein breakdown was just 1.2 g/day (2.9%) greater than synthesis.

Discussion

The current study found that habitual skeletal muscle protein FSR tended to be higher in weight-losing versus either weight-stable patients with cancer or healthy controls. These findings contradict the hypothesis that muscle wasting in cancer cachexia is caused by a reduction in protein synthesis. The current study is the first to measure skeletal muscle protein FSR during a period of days/weeks that incorporates the potentially cumulative anabolic effect of many meals over the one week protocol. As a result, there appears to be a genuine contradiction between the results of the current study and those of the previous infusion tracer studies.

In previous studies, we have demonstrated that in patients with upper gastrointestinal cancer cachexia, there is predominant downregulation of the transcriptome in human muscle consistent...
with a reduction in cell turnover/protein synthesis (10). The current study demonstrates a potential disconnect between regulatory pathways in the transcriptome and the gross level of protein synthesis in the myobiber. Studies in small animals have suggested a central role for AKT (protein kinase B) signaling and the regulation of skeletal muscle protein synthesis. However, when considered in humans, there appears to be a dissociation between cell signaling (AKT) and myofibrillar protein synthesis during feeding, exercise, and immobilization (31). No investigations of the cellular mechanisms of protein synthesis or degradation were performed during this study so it is only possible to speculate on possible explanations for the differences found between groups. One possible mechanism to account for such divergent findings is the presence of an alternative pathway sensitive to acute physiologic change/damage such as regenerative myogenesis. Indeed, muscle mass may be influenced by the balance between myofiber loss (apoptosis/autophagy) and myocyte regeneration from satellite cells. In muscle biopsies from weight-losing patients with upper gastrointestinal cancer, we have shown a threefold increase in muscle DNA fragmentation compared with control subjects. The increase in DNA laddering was associated with an increase in PARP cleavage, both observations being consistent with enhanced apoptosis (32). Concerning muscle regeneration, He and colleagues (33) have recently described in both tumor-bearing mice and patients with pancreatic cancer, activation of both satellite and nonsatellite muscle progenitor cells. However, there appeared to be a downstream block to regeneration via NFkB-mediated Pax7 dysregulation and this was thought to contribute to net muscle atrophy.

In a recent study, Williams and colleagues (3) assessed myofibrillar FSR in both the fasting and postabsorptive state. The expected increase of FSR with feeding was observed in the control subjects but was lost in patients with cancer. This finding suggests that muscle wasting in cancer cachexia could occur due to a blockade to normal anabolic stimuli and highlights the importance of considering the intermittent effects of normal stimuli to protein synthesis when estimating the overall impact of cachexia on muscle turnover over time.

An alternative explanation for the different findings between the present and previous studies may depend on the nature of the anabolic response to feeding or exercise. Conventionally FSR may be considered at basal rate representing unstimulated muscle protein turnover with periodic peaks occurring in response to feeding or exercise. In cachexia, an anabolic block could result in a simple reduction in FSR during feeding, resulting in a reduction in habitual FSR. Alternatively, instead of a reduced FSR peak with feeding, the response may be blunted and/or prolonged, possibly as the result of delayed clearance of dietary amino acids from the splanchnic bed (34).

In the current study, the median muscle loss was 5.6% per 100 days before surgery. In the group of 10 cancer patients with serial CT measurements, median body weight was 71 kg and median muscle mass was 20.2 kg (Table 3). In the patients with cancer, median myofibrillar protein synthesized per day was 41.2 g (Table 3). Median myofibrillar protein breakdown was estimated to be 42.4 g/day or 1.2 g/day (2.9%) greater than synthesis. In the context of whole body protein synthesis, myofibrillar protein may account for 20%–25%, with mixed muscle protein contributing 33%–40% of total body protein synthesis. The imbalance between breakdown and synthesis of only 2.9% is perhaps the most important observation. Such a small, but vitally important imbalance between synthesis and breakdown is difficult to quantify. Accurate assessment of the situation necessitates long-term measures of myofibrillar protein synthesis along with serial measurements of muscle mass on an individual basis. Only then will it be established whether these observations can be generalized and whether they relate to the disease stage studied.

One explanation of loss of skeletal muscle could be due to modestly increased degradation. Direct measures of skeletal muscle degradation using arteriovenous difference technology have shown increased degradation in patients with cancer compared with controls (2). Skeletal muscle synthetic and degradative pathways are closely dependent. Conventionally, this interdependence is understood to reduce synthesis when degradation is increased and vice versa (35). However, this interaction appears to be more complex, even in health where increased muscle breakdown is seen in response to the anabolic stimulus of resistance or endurance exercise (36). As such, it is possible that the changes seen in cachectic muscle reflect a process of deranged skeletal muscle remodeling where synthesis is necessarily increased but counteracted by increased muscle degradation. Alternatively, the increased synthesis could be the result of an inadequate compensatory mechanism to balance pathologic muscle breakdown. In mice, increased skeletal muscle FSR in the presence of net loss of muscle protein occurs during denervation-induced muscle wasting (37–41). The mechanism for an increase in muscle synthesis during net protein loss could be through stimulation of protein synthesis via mTORC1. This protein complex, normally associated with the maintenance of muscle mass and muscle hypertrophy could be activated by the increase in amino acids released as a result of increased protein breakdown by the proteasome (41). In a murine model of cancer cachexia, increased levels of phosphorylated Akt and increased expression of IGFI in muscle of tumor-bearing AH-130 and C26 mice suggests that muscle wasting in tumor-bearing animals may not be associated with downregulation of molecules involved in the anabolic response (42). In further support of a counter-regulatory response to compensate for muscle loss in cachexia, Op den Kamp and colleagues (43) have recently observed increased Akt phosphorylation in the muscle of patients with cachectic lung cancer. However, downstream phosphosubstrates glyoxyn synthase kinase 3β, MTORC and Forkhead box protein.

Figure 2.
Waterfall chart of preoperative skeletal muscle loss by patient as measured on cross-sectional area from sequential CT scans (during preoperative neoadjuvant chemotherapy; hashed bars, weight-stable patients; open bars, weight-losing patients). The median interval between scans was 79 days.
were unaltered and this may be evidence for overall impaired anabolic signaling. Furthermore, these complex interactions may alter with disease stage.

There are a number of potential limitations to the current study. First, the number of patients and volunteers was relatively small and matching treatment effects, diet and exercise was not possible. The small numbers resulted in groups that were not matched exactly for age (i.e., the patient group was older than the volunteer group). However, it is unlikely that a difference between the groups is masked as older individuals are likely to have a reduced FSR or reduced response to anabolic stimuli (44, 45). The patient group had undergone neoadjuvant chemotherapy. The regimens used (either cisplatin and 5-fluorouracil or epirubicin, cisplatin, and capetabine) are not known to increase protein synthesis and have previously been associated with loss of muscle mass (46) and so are unlikely to confound the results. No comparison of nutritional intake was made between patient and volunteer groups.

The possible use of nutritional supplements in the patient group could act as a stimulus to protein synthesis and the lack of an accurate record of nutritional intake represents one potential limitation of this study. With the exception of one patient who was dependent on nasogastric feeding, no patient was prescribed or reported taking oral nutritional supplements during the study period. Second, the current study used a novel protocol for the measurement of skeletal muscle FSR and it was not known at the outset whether the method would have sufficient sensitivity to distinguish between small groups. However, recent studies in healthy individuals have demonstrated an increase in myofibrillar FSR with resistance exercise in young individuals (14) and our observations reported here show a significant 11% difference in myofibrillar protein FSR between quadriceps and rectus muscles. Third, a degree of bias may arise from contamination of the myofibrillar sample from labeled amino acids from nonmyofibrillar sources such as collagen or sarcoplasmic proteins. Care was taken during this protocol to ensure no such contamination occurred. The myofibrillar isolation protocol was chosen to ensure repeated washing of the myofibrils to eliminate sarcoplasmic contamination. An additional advantage of the long protocol duration was the low precursor enrichment, which results in reduced risk of contamination of the myofibrillar fraction with labeled free amino acids. Contamination with collagen was equally unlikely; this was demonstrated by the absence of high concentrations of glycine and proline in the protein hydrolysates.

In the current study, different rates of myofibrillar protein FSR were observed in the quadriceps femoris versus the rectus abdominis muscles. This finding is consistent with previous direct measures of FSR in different muscle groups where triceps FSR differed from quadriceps FSR by approximately 15% in healthy individuals (47). The magnitude of the difference in FSR between quadriceps and rectus is, however, small and the clinical significance of the finding is uncertain. It does, however, demonstrate the importance of comparing the same muscle between subject groups and that the technique can detect small differences in FSR.

In conclusion, myofibrillar skeletal muscle FSR measured in free living individuals over the period of one week is not reduced in patients with cancer compared with healthy volunteers. These findings support the potential value of multimodal rehabilitation programs for patients with cancer that include anabolic strategies such as exercise or nutritional support to help prevent cachexia.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): A.J. MacDonald, N. Johns, A.C. Small, K.C.H. Fearon, T. Preston

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): A.J. MacDonald, J.A. Ross, A.C. Small, K.C.H. Fearon

Writing, review, and/or revision of the manuscript: A.J. MacDonald, N. Johns, N.A. Stephens, C.A. Greig, J.A. Ross, H. Husi, K.C.H. Fearon, T. Preston

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): A.J. MacDonald, N. Johns, A.C. Small, K.C.H. Fearon

Study supervision: J.A. Ross, H. Husi, K.C.H. Fearon, T. Preston

Other (obtained funding): K.C.H. Fearon

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