Autophagic-lysosomal pathway is the main proteolytic system modified in the skeletal muscle of esophageal cancer patients

Nicolas Tardif, Maria Klaude, Lars Lundell, Anders Thorell, and Olav Rooyackers

ABSTRACT
Background: In cancer cachexia, muscle depletion is related to morbidity and mortality. Muscle-wasting mechanisms in cancer patients are not fully understood.

Objective: We investigated the involvement of the proteolytic systems (proteasome, autophagic-lysosomal, calpain, and caspase) in muscle wasting during cancer cachexia.

Design: Esophageal cancer patients \( n = 14; \) mean \( \pm \) SD age: 64.1 \( \pm \) 6.6 y and weight-stable control patients undergoing reflux surgery \( n = 8; \) age: 57.5 \( \pm \) 5.8 y were included. Enzymatic activities were measured in the vastus lateralis and diaphragm. Protein expressions were also measured in the vastus lateralis of control \( n = 7 \) and cancer \( n = 8 \) patients.

Results: Proteasome, calpain, and caspase 3 activities in the vastus lateralis and diaphragm muscles did not differ between the 2 groups. Cathepsin B and L activities were 90\% \( \pm \) SD \( 2.4 \pm 0.2 \) compared with 1.3 \( \pm \) 0.2 pmol 7-amido-4-methylcoumarin (AMC) \( \cdot \) \( \mu \)g protein \( \cdot \) min \( ^{-1} \); \( P < 0.001 \) and 115\% \( 5.3 \pm 0.4 \) compared with 2.5 \( \pm \) 0.3 pmol AMC \( \cdot \) \( \mu \)g protein \( \cdot \) min \( ^{-1} \); \( P < 0.001 \) greater, respectively, in the vastus lateralis of cancer patients than in that of control subjects. We observed (in conjunction with increased lysosomal protease activities) higher microtubule-associated protein 1 light chain 3B-II/I ratios \( 0.14 \pm 0.08 \) compared with \( 0.04 \pm 0.04 \) and cathepsin B and L expressions in the vastus lateralis of cancer patients than in that of control subjects \( P < 0.05 \). Protein expression of p62 in the vastus lateralis did not differ between the 2 groups.

Conclusions: The autophagic-lysosomal pathway in the skeletal muscle of cancer patients was modified, whereas other proteolytic systems were unchanged. These findings suggest involvement of the autophagic-lysosomal proteolytic system during cancer cachexia development in humans. Am J Clin Nutr 2013;98:1485–92.

INTRODUCTION
Cancer cachexia is characterized by decreased skeletal muscle and adipose tissue mass. Nutritional support cannot reverse this body-weight loss, which leads to functional impairment. More than one-half of all cancer patients are cachectic, and this syndrome increases morbidity and mortality risks. Muscle compartment depletion seems to be particularly related to these adverse effects. Lower muscle mass has been associated with increased susceptibility to chemotherapy toxicity \( 1 \) and even with a decreased chance of survival \( 2, 3 \).

Dynamic equilibrium between anabolic (protein synthesis) and catabolic (proteolysis) processes controls muscle mass. Muscle proteolysis involves the \( U \) ubiquitin proteasome pathway \( \text{UPP} \), \( 2 \) autophagic-lysosomal pathway, \( 3 \) caspase-dependent apoptosis, and \( 4 \) calcium-dependent proteolysis pathway.

Caspases and calpain (calcium-dependent proteolysis) are believed to be involved in the initial step of myofibrillar breakdown by releasing actin and myosin filaments from the myofibrils \( 4 \). Because very few human studies have been conducted, caspases and calpains involvement in muscle wasting during cancer cachexia is still unclear.

The UPP was shown to be the most important contributor to muscle atrophy in animal models \( 5, 6 \). Three classes of enzymes are involved in the initial steps of the proteasome pathway: E1 proteins that activate ubiquitin, E2 proteins that are ubiquitin-conjugating enzymes, and E3 protein ligases that transfer ubiquitin from the conjugating system to the protein substrate \( 7 \). Three different proteases then degrade ubiquitinated proteins in the 20S core of the 26S proteasome into small peptides \( 8 \). A higher expression of the E3 ubiquitin ligases muscle-ring finger 1 \( \text{MuRF} \) 1 and atrogin 1 was observed in different experimental atrophy models, including a cancer cachexia model \( 6, 9 \). However, evidence for UPP involvement in muscle atrophy in human cancer cachexia is debatable \( 10–14 \).

Besides the UPP, it is now clear that the autophagic-lysosomal pathway is a key contributor to skeletal muscle depletion \( 15 \). In autophagy, small ubiquitin-like proteins \( \text{microtubule-associated protein 1 light chain} \text{3B} \) (LC3B), \( \gamma \)-aminobutyric-acid-type-A-receptor-associated protein (GABARAP), Golgi-associated ATPase enhancer of 16 kDa (GATE16), and autophagy-related protein 12 \( \text{Atg12} \) are involved in the formation of double-membrane vesicles \( 16 \). These vesicles (called autophagosomes)

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\small{2} Supported by Nestle Clinical Nutrition, a Society of Enteral and Parenteral Nutrition Grant, and the Swedish Cancer Foundation.

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\small{4} Abbreviations used: AMC, 7-amido-4-methylcoumarin; AU, absorbance units; DXA, dual-energy X-ray absorptiometry; LC3B, microtubule-associated protein 1 light chain 3B; MuRF 1, muscle-ring finger 1; SMI, skeletal muscle index; UPP, ubiquitin proteasome pathway.

Received April 16, 2013. Accepted for publication September 26, 2013.
First published online October 9, 2013; doi: 10.3945/ajcn.113.063859.
engulf cellular constituents and fuse with lysosomes, where their content is degraded. Cathepsins B, L, D, and H are the major proteolytic enzymes present in the lysosomes, and they primarily determine the proteolytic capacity of this organelle (17). Earlier studies observed involvement of the lysosomal proteolysis in cancer patients (13, 18). Beside these observations, very few studies investigated the role of the autophagic-lysosomal pathway in muscle depletion during cancer cachexia. However, recently, a higher expression of autophagy-related proteins in the skeletal muscle of cancer patients (10, 14) was observed, which suggests involvement of this pathway in muscle wasting. The aim of the current study was to concomitantly explore involvement of ubiquitin proteasome, autophagic-lysosomal, calpain, and caspase pathways in the vastus lateralis and diaphragm muscle in esophageal cancer patients.

SUBJECTS AND METHODS

The patients were recruited between July 2005 and May 2006. The included patients [n = 14; age (±SD): 64.1 ± 6.6 y] had received recent endoscopic and morphologic diagnoses of esophageal cancer (squamous cell cancer and adenocarcinoma) and were submitted for open surgery for primary tumor resection. No patient had been treated with preoperative radio- and/or chemotherapy. Weight-stable patients, who were undergoing laparoscopic surgery for benign, antireflux surgery (n = 8; age: 57.8 ± 5.8 y) because of gastroesophageal reflux disease, were used as the control group. The cancer patients’ body weight was measured on the day of the study. Weight change over subsequent months on the run-up to surgery was recorded (self-reports). Dual-energy X-ray absorptiometry (DXA; Lunar) measured body composition in a subgroup of cancer patients. In this subgroup of patients (n = 8), the skeletal muscle index (SMI) defined by Janssen et al (19) was used to measure sarcopenia:

\[
\text{SMI}(\%) = \frac{[\text{total body skeletal muscle mass(kg)}] \div [\text{weight(kg)}]}{100}
\]

The total-body skeletal muscle mass was computed by using the predictive equation of Kim et al (20).

Muscle biopsy specimens were collected from the vastus lateralis with a Bergstrom needle immediately after anesthesia induction and before surgery began. Samples from the diaphragm muscle were obtained during the surgical procedure, from either the right or left crus. Muscle samples were immediately frozen in liquid nitrogen and stored at −80°C until analyzed.

Protease activity measurements

Preparation of muscle sample

One-hundred milligrams of frozen vastus lateralis or diaphragm was homogenized in a glass homogenizer in 1 mL buffer A (50 mmol Tris-HCl/L, pH 7.5, 1 mmol dithiothreitol/L, 1 mmol EDTA/L, 5 mmol MgCl2/L, 250 mmol sucrose/L, and 10% glycerol). Homogenates were centrifuged at 700 × g (10 min, 4°C) to remove cell debris. The resulting supernatant fluid was then centrifuged at 16300 × g (20 min, 4°C). The supernatant fluid was frozen in aliquots at −80°C and used for determination of proteasome, calpain, and caspase 3 activities. The pellet was rinsed in 1 mL buffer B (0.15 mol KCl/L and 20 mmol sodium phosphate/L, pH 6.0) and then resuspended in 150 μL buffer B. The pellet suspension, which contained lysosomes, was frozen in aliquots at −80°C and used to determine cathepsin activities. Before the measurement of protease activities in the 2 fractions, the protein content was measured by using the BioRad protein assay, based on Bradford’s method. Just before the analyses, the pellet suspension was first freeze-thawed 3 times.

Proteasome activity

Chymotrypsin-like activity of the proteasome fraction was measured by using the fluorogenic substrate SUC-LLVY-AMC [succinyl-Leu-Val-Tyr-(7-amido-4-methylcoumarin; AMC); Sigma-Aldrich] (21). Ten microliters of the supernatant fluid (~10 μg protein) was incubated in 100 μL buffer (50 mmol Tris-HCl/L, pH 7.5, 1 mmol ATP/L, 5 mmol MgCl2/L, 1 mmol/L, and 150 μmol LLVY/L) in microplates. Standard curves were prepared by using the AMC. Fluorescence was measured continuously over 1 h at 37°C in a FLOUstar OPTIMA spectrofluorometer (BMG Laboratory Technologies) at λex = 380 nm and λem = 460 nm. Proteolytic activity was calculated from the increment of the curves from samples and standards and is expressed as pmol of the AMC released/μg protein per minute.

Cathepsin activities

Lysosomal fraction activity was measured by using Z-Arg-Arg-AMC for cathepsin B and Z-Phe-Arg-AMC for cathepsin L (Sigma-Aldrich) (22, 23). For cathepsin B activity, 10 μL pellet suspension (~2 μg protein) was incubated in 100 μL buffer (0.1 mol sodium phosphate/L, pH 6.0, 1 mmol EDTA/L, 2 mmol cysteine/L, and 250 μmol Arg-Arg-AMC/L). For cathepsin L activity, 10 μL pellet suspension was incubated in 100 μL buffer (0.1 mol sodium acetate/L, pH 5.5, 1 mmol EDTA/L, 1 mmol dithiothreitol/L, and 250 μmol Phe-Arg-AMC/L). Standard curves were prepared by using AMC. Fluorescence and proteolytic activities were measured and calculated as described for proteasome activity.

Caspase 3 activity

Activity was measured by using Ac-DEVD-AMC (N-acetyl-Asp-Glu-Val-Asp-AMC; Sigma-Aldrich) as substrate (23). Supernatant fluid aliquots (30 μg protein) were incubated in 100 μL reaction buffer (100 mmol HEPES/L, pH 7.5, 10% sucrose, and 1 mmol dithiothreitol/L) that contained 100 μmol DEVD-AMC/L at 30°C for 1 h. Parallel incubations were done in the presence of 30 μmol Ac-DEVD-CHO/L (N-acetyl-Asp-Glu-Val-Asp aldehyde; Sigma-Aldrich)—a caspase 3 inhibitor. Fluorescence and proteolytic activity were measured and calculated as described for proteasome activity. Activity in the presence of Ac-DEVD-CHO was subtracted from the activity in the presence of Ac-DEVD-AMC alone and expressed as fluorescence units/μg of protein per minute.

Calpain activity

Calpain activity in the supernatant fluid was determined by using BODIPY FL-casein (4,4-difluoro-5,7-dimethyl-4-boronic acid-3a,4a-diaza-s-indacene-3-propionic acid-labeled casein; molecular probes) as substrate (23). Then, 30 μg supernatant fluid protein was incubated in a buffer that contained 20 mmol Tris-HCl/L, pH 7.5, 1 mmol EDTA/L, 100 mmol KCl/L, 1 mmol dithiothreitol/L, 10
mmol CaCl₂/L, and 4.6 μg BODIPY FL-casein/mL in a total volume of 220 μL. Parallel incubations were done without calcium and in the presence of 10 mmol EDTA/L. Fluorescence was measured continuously over 1 h at 26°C at λex = 485 nm and λem = 520 nm. Proteolytic activity was calculated from the increment of the curves obtained and expressed as fluorescence units/μg protein per min. By subtracting the activity curve without calcium, the calcium-dependent activity, that is, calpain activity was obtained.

Western blot

Because all diaphragm biopsies were completely used for the proteolysis activity measurements, Western blot analyses were only done in the vastus lateralis, where 8 biopsies remained for the cancer group and 7 for the control group. Fifty milligrams of frozen vastus lateralis was minced and homogenized in a Mini-Beadbeater (BioSpec Products) in an ice-cold buffer (50 mmol Tris-HCl/L, pH 7.6, 150 mmol NaCl/L, 10 mmol EDTA/L, 10 mmol Na₂PO₄/L, 25 mmol β-glycerophosphate/L, 100 mmol NaF/L, 2 mmol sodium orthovanadate/L, 10% glycerol, and 1% Triton X-100) that contained 1% of protease inhibitor cocktail (Sigma-Aldrich). Homogenates were centrifuged at 12,000 × g for 10 min at 4°C to remove cell debris. Denatured proteins (20 μg) were separated by using SDS-PAGE on a gradient polyacrylamide (4–20%) precast gel (no. 25249; Pierce). Proteins were blotted on a polyvinylidene membrane (immobilon-FL; Millipore). Immunoblots were blocked 1 h with a blocking buffer (LI-COR Biosciences) and then probed with primary antibodies: anti-MuRF1 (1/1000, no. NBP1-54939; Novus Biologicals), anti-atrogin-1 (1/1000, no. AP2041; ECM Biosciences), anti-cathepsin B (1/200, no. IM27L; Calbiochem), anti-cathepsin L (1/200, no. C2970; Sigma-Aldrich), anti-LC3 (1/100, no. 0231–100; Nanotools), and anti-p62 (1/2000, no. H00008878-M01; Abnova). After several washes in PBS plus 0.1% Tween 20, immunoblots were incubated with an IRDye 680LT or an IRDye 800CW secondary antibody (LI-COR Biosciences). Membranes were scanned on an Odyssey scan (LI-COR Biosciences). After these steps, membranes were stained with Coomassie blue (PhastGel blue R-350; GE Health Care), and total protein measured with ImageJ software (NIH) was used as a loading control as previously described (24).

Statistical methods

All data were analyzed by using Statistica 8 (StatSoft France). Student’s t test was used for comparisons between cancer patients and control subjects after normal distribution data were confirmed by using the Shapiro-Wilk test. Results are given as means ± SDs. Correlation were assessed with the Spearman correlation test.

RESULTS

Clinical data

Patient characteristics are shown in Table 1. Cancer patients were older and had lower BMIs in comparison with control subjects (P < 0.05). There was a trend toward lower albumin concentrations in the cancer group than in the control group (P = 0.06). However, mean albumin concentrations in cancer patients were not under the hypoalbuminemia threshold of 35 g/L (25). Esophageal cancer patients reported a wide range of weight loss before surgery, from no weight loss to loss of 29.3% of total body weight (median: 1.7% weight loss per month). In the 8 patients undergoing a DXA scan, monthly weight loss was significantly correlated with leg muscle mass (R = −0.79, P = 0.02; n = 8; Figure 1A) but not with fat mass (R = −0.20, P = 0.63; n = 8; Figure 1B). Clinical data for the DXA subgroup of patients are shown in Table 1. In this subgroup of patients, 5 were sarcopenic (SMI <37%) and 3 had normal muscle mass (SMI >37%).

| Table 1 Clinical data for the esophageal cancer patients and control subjects |
|-----------------------------|---------------------------|---------------------|
| Age (y)                     | Sex: Female               | Male                |
| Control                     | 57.5 ± 5.8                | 61.4 ± 6.6          | 62.9 ± 4.9          |
| Cancer                      | 52.3 ± 5.5                | 63.6 ± 4.5          | 64.8 ± 4.4          |
| Total-body weight loss (%)  | NA                        | 7.0 (0–29.3)        | 7.5 (0–29.3)        |
| Weight loss rate (%/mo)     | NA                        | 1.7 (0–7.2)         | 1.7 (0–2.4)         |
| Tumor site                  | Middle third              | Distal third        | Not commented       |
| Control                     | 1                         | 11                  | 2                   |
| Cancer                      | 0                         | 6                   | 2                   |
| Histology                   | Adenocarcinoma            | Squamous cell carcinoma | Indeterminate |
| Control                     | 7                         | 5                   | 1                   |
| Cancer                      | 4                         | 3                   | 1                   |
| Grade                       | High grade                | Low grade           | Not specified       |
| Control                     | 3                         | 9                   | 2                   |
| Cancer                      | 3                         | 4                   | 1                   |

1 An unpaired t test was used to assess differences between the control and cancer patients; *P < 0.01. The DXA-derived clinical data for the subgroups did not differ from the cancer group. DXA, dual-energy X-ray absorptiometry; NA, not assessed.

2 DXA-derived body-composition data for the cancer patients.

3 Mean ± SD (all such values).

4 Median; range in parentheses (all such values).

Proteasome activity

In the vastus lateralis, no differences occurred in 20S proteasome activity, that is, calpain activity was obtained.

Autophagic-lysosomal proteolysis

LC3B is a protein involved in autophagosome formation. Autophagy activation leads to conversion of the cytosolic-soluble LC3B-I to its lipiddated autophagosome-bound form: LC3B-II. The LC3B-II/I ratio reflects the number of autophagosomes (26).
A higher L3CB-II/I ratio was recorded in the vastus lateralis of cancer patients than of the control subjects (0.14 ± 0.08 compared with 0.04 ± 0.04; P < 0.05; Figure 3A), which indicates increased autophagosomes.

The p62 (also called sequestosome 1) protein recognizes ubiquinated substrates and transports them to the autophagosome via its interaction with LC3B-II. When autophagy is suppressed, p62 accumulation can be monitored by using Western blot (27). We did not observe higher p62 protein expression in the vastus lateralis in the cancer group [2.4 ± 0.6 compared with 2.2 ± 0.2 absorbance units (AU); cancer compared with control patients; Figure 3B]. Together with the higher LC3B-II/I ratio, this observation suggested that there was no impairment of autophagosome maturation in the skeletal muscle of patients with esophageal cancer.

The catalytic unit (autolysosome) mediates the final step of the autophagic-lysosomal pathway. In agreement with increased autophagy, we observed 60% higher cathepsin B activity in the diaphragm and 90% higher cathepsin B activity in the vastus lateralis of cancer patients than of control patients (Table 2). Significantly higher cathepsin L activity was also higher in the diaphragm (>60%) and vastus lateralis (115%) of cancer patients than of control subjects (Table 2). No correlations were observed between weight-loss rate and cathepsin B activity (r = 0.33, P = 0.25) or cathepsin L activity (r = 0.48, P = 0.08).

These higher cathepsin activities were associated with higher expression of the mature form of cathepsin B (22.1 ± 11.0 compared with 12.0 ± 5.0 AU; cancer group compared with control group; P < 0.05; Figure 3C) and cathepsin L (7.4 ± 2.0 compared with 4.5 ± 2.7 AU, cancer group compared with control group; P < 0.05; Figure 3D).

Calpain and caspase 3 pathways

Our results showed no difference between cancer patients and control subjects when it comes to caspase 3 activity in the vastus

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**FIGURE 1.** Correlation between body composition evaluated by dual-energy X-ray absorptiometry and the rate of body-weight loss in esophageal cancer patients. A: Correlation between leg-muscle mass and body-weight loss per month (R = −0.79, P = 0.02). B: Correlation between body-fat mass and body-weight loss (R = −0.20, P = 0.63). Correlations were assessed with the Spearman correlation test.

**FIGURE 2.** Mean (± SD) E3 ligase protein expression in the vastus lateralis of control (n = 7) and cancer (n = 8) patients. Normal distribution of the values was assessed with the Shapiro-Wilk test. Differences between the control and cancer patients were assessed with an unpaired t test. No significant differences were observed in MuRF 1 (A) and atrogin 1 (B) protein expression in the vastus lateralis of control and cancer patients. Representative blots of MuRF 1 and atrogin 1 expression (C). The total amount of protein per lane was used as a loading control, as previously described (24). The amount of protein was measured by using densitometry of the Coomassie-stained membrane (C). AU, absorbance units; MuRF 1, muscle-ring finger 1.
lateralis or diaphragm (Table 2). Experimental models of cancer cachexia showed increased calpain activity (28) or no change in this proteolytic activity (29). In the current study, we did not observe differences between the 2 groups in calpain activity in the vastus lateralis (Table 2). Calpain activity in the diaphragm was under the limit of detection of our enzymatic measurement, so it could not be measured in this muscle.

**DISCUSSION**

We observed autophagic-lysosomal pathway activation in the skeletal muscle of esophageal cancer patients without activation of the other proteolytic pathways (UPP, caspase, and calpain). Increased proteasome activity was associated with muscle wasting in animal models of cancer (29–31). In humans, one study showed higher proteasome activity in the skeletal muscle of cancer patients compared with controls (29). The current study showed no difference in proteasome activity between cancer patients and control patients.

**TABLE 2**

Proteolytic enzyme activities in the skeletal muscle of esophageal cancer patients and control patients

<table>
<thead>
<tr>
<th></th>
<th>Diaphragm</th>
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<th>Vastus lateralis</th>
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<tr>
<td></td>
<td>Control (n = 8)</td>
<td>Cancer (n = 14)</td>
<td>Control (n = 8)</td>
<td>Cancer (n = 14)</td>
</tr>
<tr>
<td>Caspase 3 (fluorescence unit · µg⁻¹ · min⁻¹)²</td>
<td>2.25 ± 0.78</td>
<td>2.49 ± 0.98</td>
<td>7.90 ± 3.03</td>
<td>9.02 ± 2.22</td>
</tr>
<tr>
<td>Calpains (fluorescence unit · µg⁻¹ · min⁻¹)²</td>
<td>ND</td>
<td>ND</td>
<td>3.37 ± 1.00</td>
<td>3.93 ± 1.03</td>
</tr>
<tr>
<td>20S proteasome (pmol AMC · µg protein⁻¹ · min⁻¹)</td>
<td>0.44 ± 0.21</td>
<td>0.54 ± 0.23</td>
<td>0.69 ± 0.17</td>
<td>0.80 ± 0.17</td>
</tr>
<tr>
<td>Cathepsin B (pmol AMC · µg protein⁻¹ · min⁻¹)</td>
<td>1.02 ± 0.54</td>
<td>1.60 ± 0.54*</td>
<td>1.25 ± 0.52</td>
<td>2.37 ± 0.74*</td>
</tr>
<tr>
<td>Cathepsin L (pmol AMC · µg protein⁻¹ · min⁻¹)</td>
<td>2.21 ± 1.23</td>
<td>3.57 ± 1.23*</td>
<td>2.47 ± 0.84</td>
<td>5.32 ± 1.47**</td>
</tr>
</tbody>
</table>

¹All values are means ± SDs. Differences between the control and cancer patients were assessed by using an unpaired *t* test: *P < 0.05, **P < 0.001.

²Calpain and caspase 3 activities were calculated in the presence or absence of inhibitors.

**FIGURE 3.** Mean (± SD) expression of protein of the autophagic-lysosome pathway in the vastus lateralis of control (n = 7) and cancer (n = 8) patients. Normal distribution of the values was assessed with the Shapiro-Wilk test. Differences between the control and cancer patients were assessed with an unpaired *t* test: *P < 0.05 compared with the control group. Protein expression of LC3B-II/LC3B-I (A), p62 (B), cathepsin B (C), and L (D). Representative blots of protein expression (E). The total amount of protein per lane was used as a loading control, as previously described (24). The amount of protein was measured by using densitometry of the Coomassie-stained membrane (E). AU, absorbance units; Cath, cathepsin; LC3B, microtubule-associated protein 1 light chain 3B; SQSTM1, sequestosome 1.
of gastric cancer patients (11), whereas this was not observed in lung cancer patients (12).

Disease severity might explain the discrepancies between these studies. Bossola et al (11) observed a 270% increase of proteasome activity in the skeletal muscle of stage IV patients compared with stage I-II patients. The study by Op den Kamp et al (12) included cancer patients with low tumor grade (as in the current study), which may explain the differences between these studies.

Related to the proteasome activity, 2 E3 ligases, MuRF 1, and atrogin 1 increased in several experimental muscle atrophy models (9). Increased gene expression of these E3 ligases was particularly observed in tumor-bearing animals (6). The difference between animal models and human cancer patients was the development and severity of the tumor burden. In animals, the tumor could reach up to 30% of the body weight and cause massive, rapid body-weight loss. In humans, the tumor generally reaches <1% of total body weight. The proteasome pathway seems to be activated in muscle during late stages of cancer (11, 32) or in acute weight-loss conditions, such as sepsis (21, 23). Here, results from animal studies may be more related to acute weight-loss conditions or to late cancer cachexia stages.

Other proteolytic systems should be activated during muscle-wasting development in cancer cachexia cases (12, 32); calpain, caspase, and autophagic-lysosome could be the first triggers. Two clinical studies estimated apoptosis in the skeletal muscle of cancer patients, which reported higher apoptosis in gastrointestinal cancer patients (33), whereas this could not be confirmed in the other study (34). In these studies, the disease stage was the same and thus could not explain this discrepancy. In the study by Bossola et al (34), apoptosis was assessed by using several methods, including the terminal deoxynucleotidyl transferase (TdT) dUTP nick-end labeling (TUNEL) assay; the study found no change between these markers. In the current study, we observed no difference in caspase 3 activity, which might indicate that apoptosis does not occur in our cancer patients’ skeletal muscles.

No change in calpain activity was observed, which indicates no calcium-dependent proteolytic pathway involvement. To the best of our knowledge, this study was the first to measure calpain activity in cancer patients’ skeletal muscles. In the skeletal muscles of tumor-bearing rats (Yoshida AH-130), decreased calpastatin (endogenous calpain inhibitor) activity was observed without changed calpain activity (35). The calpain/calpastatin activity ratio could be a key factor during calcium-dependent proteolysis assessment. Because we did not measure calpastatin activity, we could not draw definitive conclusions regarding calcium-dependent proteolysis involvement.

Autophagic-lysosomal pathway is important for muscle mass control. Overactivation or impairment of the autophagic-lysosomal system can induce muscle wasting (15). Thus, it is crucial to be able to distinguish between increased autophagic-lysosomal proteolysis and an autophagic process blockade. In genetic diseases, such as Danon and Pompe diseases, a blockade of the fusion between the autophagosomes and the lysosome leads to accumulation of autophagosomes in the cell, which induces muscle dystrophy (36). Accumulation of p62 is a surrogate marker of autophagy inhibition (29). In the current study, we did not observe higher p62 expression despite an increase in the LC3B-II/I ratio. Instead, these observations suggest increased autophagy processes rather than an autophagy blockade. A recent study that measured increased autophagy flux during the initial muscle-wasting stage in C26-bearing mice (37) appears to confirm this conclusion.

The catalytic unit (autolysosome) mediates the final step of the autophagic-lysosomal pathway. In humans, earlier studies suggest the involvement of the lysosomal pathway during cancer cachexia (13, 17). Higher lysosomal activities (cathepsin D and acid phosphatase) (17) and cathepsin B mRNA expression (13) were observed in the skeletal muscle of cancer patients. Compared with previous studies, higher cathepsin B and L expression and higher cathepsin activity were measured in the vastus lateralis of cancer patients in the current study. Here, the higher cathepsin expressions and activities were associated with changes in autophagy-related protein expression. This indicates activation of the autophagic-lysosomal pathway in the skeletal muscle of cancer patients.

Esophageal cancer patients experience decreased food intake because of dysphagia. Because starvation has been shown to trigger autophagy flux in the skeletal muscles of mice (38), our results might be explained by a lower dietary intake because of the specific esophageal cancer pathology. Unfortunately, we could not collect dietary intake data. However, earlier studies showed that the ATP-ubiquitin-dependent proteolytic system is the major proteolytic pathway that is activated by fasting in animals (39, 40). In direct comparison of the effect of starvation and cancer in incubated muscle, higher cathepsin activities were observed only in the tumor-bearing rats and not in the starved rats, when compared with controls (41). Thus, we suggest that the observed higher cathepsin activities are more related to the cancer effect than solely to a semistarvation process.

In a recent human study, Deans et al (42) investigated the influence of several factors on weight loss in esophageal cancer patients. They observed the same range of body-weight loss that we observed (2.5% body-weight loss per month). Multiple regression analysis identified 3 independent variables related to this weight loss: 1) dietary intake, 2) inflammation status (serum C-reactive protein concentrations), and 3) disease stage, which explained 38%, 34%, and 28%, respectively, of the body-weight loss in these patients (42). Reduced energy intake was also correlated with fat-mass loss but not with muscle-mass loss in weight-losing cancer patients (43). In elderly patients, inflammation proved to be strongly associated with muscle-weight loss (44) and was also reported to be a good predictor of lean mass in palliative cancer patients (45). Inflammatory processes might explain the negative correlation between leg-muscle mass (not fat mass) and body-weight loss that we observed. However, more complete data on body composition and our patients’ inflammation status were not available to validate this observation. In lung cancer patients, inflammation (local and systemic) has been associated with an increase in muscle atrophy and higher autophagy marker expression. This observation, made in other types of cachectic cancer patients, supports the results of our study (14).

Our study was limited by the small number of patients included and the even smaller numbers of Western blot analyses and DXA measurements. Thus, we had limited power to detect any correlations between weight loss and the enzyme activities. Larger studies are needed to address this issue. Because our primary aim was to measure enzyme activities, protein expression was measured merely to confirm our primary results.
In conclusion, the current study simultaneously measured the activities of the 4 main proteolytic systems in the leg muscle (vastus lateralis) and respiratory muscle (diaphragm) of cancer patients. However, unlike in animal studies, UPP was not activated. This discrepancy highlights the need for clinical studies to investigate cancer cachexia mechanisms in humans. Although all proteolytic systems were investigated, the only changes observed were in the autophagic-lysosomal system. This suggests that the autophagic-lysosomal proteolytic system is activated and involved in the development of human cancer cachexia.

The authors’ responsibilities were as follows—NT, MK, and OR: designed and conducted the research and had primary responsibility for the final content; LL and AT: contributed to the project development, recruited the participants, performed the surgical procedures, and conducted the clinical supervision of the patients; and NT: analyzed the data, performed the statistical analysis, and wrote the manuscript. All authors interpreted the results, critically reviewed the manuscript drafts, and approved the final manuscript. None of the authors stated any conflicts of interest.

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