A new model of cancer cachexia: contribution of the ubiquitin-proteasome pathway

DOUGLAS D. LAZARUS,1 ANTONIA T. DESTREE,2 LAUREEN M. MAZZOLA,1 TERESA A. MCCORMACK,1 LAWRENCE R. DICK,1 BI XU,1 JIAN Q. HUANG,1 JACQUELINE W. PIERCE,1 MARGARET A. READ,1 MICHAEL B. COGGINS,1 VERED SOLOMON,2 ALFRED L. GOLDBERG,2 STEPHEN J. BRAND,1 AND PETER J. ELLIOTT1

1ProScript, Cambridge 02139; and 2Department of Cell Biology, Harvard Medical School, Boston, Massachusetts 02115


THE TERM CACHEXIA refers to the dramatic weight loss that is characteristic of several systemic diseases, especially diabetes, acquired immunodeficiency syndrome, and cancer (18, 21, 38). Cancer cachexia, a slowly developing wasting syndrome, is unrelenting in the face of current nutritional and drug therapies (19, 37). Many cancer patients exhibit unexplained weight loss as the first symptom of the disease, and nearly all cancer patients have lost significant weight by the time of death (9, 36). Although a decline in appetite is often associated with cancer cachexia (14), weight loss in this condition is not reversed by an increased food intake (19, 41).

The pathological consequences of cachexia result primarily from a loss of muscle tissue (19, 37). It is believed the severe protein malnutrition that develops leads to a compromised immune system and increased susceptibility to infection (4). The accompanying weakness and malaise result in a lower quality of life (9, 14, 38) and are associated with poor tolerance to anticancer therapy (1, 9, 23, 26, 38). To date, no effective treatment of this cachectic state has been developed.

Although experimental models of cancer cachexia are known that appear to mimic human cachexia, the pathways that cause cachexia remain poorly understood. As with human cachexia, in such animal models there is a significant loss of adipose and muscle tissue, and the animals develop substantial hypoglycemia despite a normal food intake (3, 6, 29, 48, 56, 57).

Principal among the major circulating cytokines implicated in models of cachexia are tumor necrosis factor (TNF)-α and interleukin (IL)-6 (49). For example, circulating TNF-α is elevated in the cachectic rats bearing the Yoshida hepatoma ascites tumor and contributes to the resulting muscle loss (7, 54). Similarly, IL-6 is important in the weight loss that develops in mice bearing the colon-26 (C-26) tumor (12, 31, 48, 50, 63). Nevertheless, definitive proof of a role for either TNF-α or IL-6 in cancer weight loss remains elusive. Although anti-TNF-α antibody treatment decreased muscle protein turnover in Yoshida hepatoma-bearing rats, the weight loss was not affected (7, 54). In mice bearing the C-26 tumor, several agents attenuate the weight loss, including IL-1 receptor antagonist, IL-12, dexamethasone, and indomethacin, all of which appeared to act by interrupting IL-6 secretion (12, 31, 50, 63). However, mice bearing a C-26-derived tumor that did not cause weight loss had similarly elevated circulating IL-6 levels (43). This finding implies that factor(s) other than TNF-α or IL-6 were more important in these models of cancer cachexia. Further, these observations suggest that cytokine-dependent tumor models of cachexia may not truly reflect the human cancer cachexia and, due to their critical reliance on cytokines, could be used to develop treatments that are destined to fail in the clinic.

Experimental systems are important not only for the studies of the specific inducers of cachexia but also for the understanding of the biochemical pathway through...
which muscle protein loss develops. The muscle wasting that characterizes cachexia appears to be due largely to increased protein degradation. For example, in cachectic rats bearing the Yoshida hepatoma AH-130 ascites tumor, muscle protein breakdown increased two- to threefold, exceeding protein synthesis and leading to net protein loss (55). There are several proteolytic pathways in skeletal muscle. Of these systems, the ATP-dependent ubiquitin-proteasome pathway is of major importance in conditions where there is a general loss of muscle mass (33, 34, 55, 58, 61). In cachectic mice and rats, the mRNA of several key proteins in the ubiquitin-proteasome pathway, including ubiquitin, proteasome subunits, and 14-kDa E2 ubiquitin-conjugating enzyme (E214k), were elevated (27, 33, 47, 53). In addition, cellular levels of ubiquitin and ubiquitin-protein conjugates increased (24, 27, 33).

To develop the tumor model used in the studies described here, clones of the C-26 tumor were isolated and implanted into mice. A clone that induces a severe cachexia, termed R-1, was employed in the subsequent investigations. In R-1 tumor-bearing mice, food intake did not decline, even when weight loss was >20% of initial body weight. With this cancer cachexia, hypoglycemia developed and losses of both adipose and muscle tissues became considerable. After the physiological responses were defined, we investigated the role of the ubiquitin-proteasome pathway in the proteolysis in muscles from nontumor-bearing controls and cachectic mice. Data presented here further support a role for the ubiquitin-proteasome pathway in cachexia. Amino acid release by incubated skeletal muscle increased in cachectic mice, compared with controls. Moreover, the elevated proteolysis from cachectic muscles was suppressed by a specific proteasome inhibitor and was associated with an enhanced rate of ubiquitin conjugation.

This novel cytokine (non-IL-1β, IL-6, and TNF-α)-independent model of cachexia offers a new opportunity for investigating the pathway(s) by which tissue wasting develops in cancer patients and provides a system in which relevant therapies can be evaluated.

**METHODS**

**Materials**

Except where noted, the reagents used were purchased from Sigma (St. Louis, MO). High glucose DMEM containing L-glutamine, RPMI 1640, and fetal bovine serum (FBS) were from J RH Biosciences (Lenexa, KS). Metofane was from Henry Schein (Port Washington, NY), and 125I-iodine was from New England Nuclear (Boston, MA). IL-1β, IL-6, and TNF-α ELISA were obtained from Endogen (Woburn, MA) or BioSource International (Camarillo, CA). The Coomassie Plus protein assay was from Pierce (Rockford, IL). Rabbit anti-E214k antibody and rabbit anti-ubiquitin antibody were the kind gifts of Simon Wing (McGill University, Montreal, QC, Canada) and Cecile Pikart (Johns Hopkins University, Baltimore, MD), respectively. The coding region of E214k was obtained by PCR from human Jurkat cell cDNA. Lactacycin was synthesized at ProScript, Cambridge, MA.

**Mice**

Male CD2-F1 (BALB/c x DBA2, 22–30 g) mice were used in this study (Taconic Farms, Germantown, NY). Male BALB/c nude mice were obtained from the National Cancer Institute (Frederick, MD). The mice were housed 3–5 per cage in a 12:12-h light-dark cycle, with access to standard laboratory chow (Purina Mills, St. Louis, MO) and water ad libitum.

**C-26 Tumor Clone Isolation**

The original parent tumor was obtained from the National Cancer Institute (Frederick, MD). After several passages in mice, 41 clones were isolated after mechanical separation of a tumor through a Collecter wire gauze (VWR Scientific, Boston, MA) and grown in RPMI 1640 medium containing 10% FBS and penicillin-streptomycin antibiotics (50 U/ml and 50 µg/ml, respectively). After isolation, the clones were grown in high glucose DMEM with similar additives.

**To determine the cachexia-inducing effects of each clone,** 1 × 106 cells in 100 µl sterile phosphate-buffered saline were implanted intradermally in the right flank of male CD2-F1 mice. Body weight and tumor size were monitored daily. A clone that induced cachexia, termed R-1, was used subsequently in 13 independent studies.

**Cachexia in Tumor-Bearing Mice**

Passages 9 to 11 of the R-1 clone were implanted intradermally into male CD2-F1 mice, 5 × 103 cells in 100 µl of PBS. Body weights and food intake were monitored. Serum was obtained for analyses from blood collected by cardiac puncture of mice anesthetized with Metofane inhalation. The right epididymal fat pad was used to measure changes in adipose tissue (12, 48). The muscles removed for comparison were the extensor digitorum longus (EDL), gastrocnemius, and soleus muscle. The control groups consisted of nontumor-bearing mice of the same age.

**Drug Treatments**

Dexamethasone 21-phosphate was dissolved in 0.9% saline and given as 33, 100, or 300 mg/kg. Indomethacin was dissolved in 0.2 M Trizma base and given at 0.7, 2.0, or 6.0 mg/kg. All treatments were given intraperitoneally in a volume of 100 µl/20 g mouse. Age-matched controls were given the corresponding vehicle.

**Protein Assay**

EDL muscles were homogenized in a 10-fold volume of PBS and centrifuged at 16,000 g for 20 min. The supernatant protein content represented soluble cellular protein, and the pellet represented the insoluble protein content, including the myofibrillar protein (22). The pellet was dissolved in 0.1 M sodium hydroxide before protein measurement. Protein measurement was done with the Coomassie Plus protein assay. BSA was used as the protein standard (Pierce).

**E214k mRNA Analysis**

Total RNA was isolated from gastrocnemius muscles by the TRIzol reagent method (Life Technologies, Gaithersburg, MD). RNA (10 µg) was separated by electrophoresis on a 1% agarose formaldehyde gel, transferred to Hybond-N membrane (Amersham, Arlington Heights, IL) and immobilized by ultraviolet radiation with an ultraviolet Stratalinker 2400 (Stratagene, La Jolla, CA). Blots were prehybridized for 4 h and hybridized overnight at 65°C with 32P-labeled DNA probes (MegaPrime kit, Amersham). The band densitometry...
was determined with a Storm 840 PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

**Immunoblotting**

To measure the E214k protein and ubiquitin-protein conjugate levels in gastrocnemius muscles, 0.1 g of tissue was homogenized with 300 µl of buffer containing 50 mM HEPES, 1 mM dithiothreitol, and 100 mM NaCl. The homogenate was centrifuged at 14,000 g for 20 min at 4°C, and the supernatant was passed through a 22-µm filter. For each sample, 20 µg of soluble protein were analyzed by quantitative Western blot. Ubiquitin-conjugates were analyzed relative to soluble protein levels with rabbit anti-ubiquitin and Amersham enhanced chemiluminescence detection. E214k was analyzed by quantitative Western blot, with rabbit anti-E214k antibody. The E214k immunoblot was developed with the Vistra AutoPhos system (Amersham Life Sciences). The developed blot was scanned with a Storm 840 PhosphorImager.

**Proteasome Activity**

Gastrocnemius muscle 20S proteasome activity was measured with the fluorogenic substrate succinyl-Leu-Leu-Val-Tyr-7-amino-4-methylcoumarin (47).

**Muscle Ubiquitination Activity**

Fraction II muscle extracts were prepared from 4 to 5 gastrocnemius muscles, as described earlier (44). Rates of protein conjugation by ubiquitin and ubiquitin conjugation of the substrates lysozyme and α-lactalbumin were done as described earlier (44).

**EDL Incubation and Amino Acid Release**

After surgical removal, the EDL muscles were tied with thread at each end and maintained in the stretched position (1.1 cm in length). The muscles were preincubated for 30 min at 38°C in Krebs-Ringer buffer containing HEPES, pH 7.2–7.4, and the treatments (see below) were in a 95% air-5% CO2 mixture. The incubations were carried out in a shaking water bath at 80 cycles/min. After equilibration, each EDL muscle was placed into another flask with similar conditions for 4 h, after which buffer and muscles were collected for analyses. Methionine, phenylalanine, and tyrosine were measured. Prior work with EDL muscles showed that amino acid release is linear for 4 h (data not shown).

The incubation medium contained 2 mM HEPES, 135 mM NaCl, 5 mM KCl, 2 mM CaCl2, 1 mM MgCl2, 1 mM Na2HPO4, 15 mM NaHCO3, 11 mM glucose, 0.1 U/ml insulin, 0.1 mM isoleucine, 0.17 mM leucine, and 0.2 mM valine. Cycloheximide (0.5 mM) was added to inhibit protein synthesis, methylamine (10 mM) was added to inhibit lysosomal proteolysis, and E64d (50 µM) was added to inhibit lysosomal and calpain-mediated proteolysis. For each muscle exposed to lactacystin (100 µM), the contralateral muscle from the same animal was exposed to the vehicle (DMSO, final concentration 0.1%).

**Amino Acid Analysis**

With the use of a method previously described (5), 20 µl of EDL incubation medium were mixed with 60 µl of 0.2 M sodium borate buffer (pH 10) and 20 µl AccQ-Fluor reagent (Waters, Milford, PA). This mixture was heated at 55°C for 10 min. Then, 30 µl of AccQ-tag buffer were added. After the samples were filtered, this solution was analyzed on an HPLC (Waters, Milford, PA) with a C18 reverse-phase column and an isocratic elution of 79% 20 mM sodium phosphate, pH 7.2-21% acetonitrile. We also prepared the incubation medium buffer that was not incubated with EDL muscles to correct for background fluorescence. The fluorescence wavelengths used were 365 nm excitation/460 nm emission.

**Statistics**

All data are presented as means ± SE. Groups were compared with ANOVA, with the post hoc Dunnett’s multiple range test or unpaired t-test, as appropriate.

**RESULTS**

The Novel R-1 Model of Cachexia

After tumor implantation, body weight was stable until day 10. At this time, tumor weights were 0.30 ± Fig. 1. A: body weights of control and R-1 tumor-bearing mice, n = 6 and 12, respectively. B: food intake of mice whose body weights are illustrated in A. Weights of tumor-bearing mice were significantly less than control mice from day 11 to day 17 posttumor implantation, *P < 0.05.
0.08 g. Thereafter, weight loss declined up to 5% per day (Fig. 1A). The loss of body weight became statistically significant on day 11, $P < 0.05$ vs. controls, and remained so until day 17. On day 17, the body weights of the control group were $30.1 \pm 0.7$ g, whereas for the tumor-bearing group they were $22.0 \pm 0.5$ g when the weight of the tumor was subtracted. The nontumor body weights of R-1 tumor-bearing animals represented only 73% of the body weights of control animals. At this time, i.e., on day 17 posttumor implantation, the tumor weights were $1.51 \pm 0.12$ g, or 6% of body weights. Importantly, although significant body weight loss occurred, food intake was not affected in these tumor-bearing mice (Fig. 1B).

Circulating glucose concentrations decreased during the cachexia, declining to roughly 30% of those in the age-matched controls (Table 1). The epididymal fat pad weights in cachectic mice, declared as body weight declined and were significantly lower on day 17 (Table 1). At this time, the adipose tissue was only 5% of that removed from age-matched control animals. The weights of the EDL, gastrocnemius, and soleus muscles declined significantly during the study and were 15–20% lower than those removed from the control group (Table 1).

EDL muscle-soluble protein content was only 5% of that removed from age-matched control animals. The weights of the EDL, gastrocnemius, and soleus muscles declined significantly during the study and were 15–20% lower than those removed from the control group (Table 1).

EDL muscle-soluble protein content was not lower in cachectic mice, even though they lacked mature T lymphocytes. Body weights, blood glucose, epididymal fat pad weights, and muscle weights declined significantly in tumor-bearing nude mice, $P < 0.05$ vs. the nontumor-bearing control group. The cytokines IL-1β, IL-6, and TNF-α were also monitored, and no reliable levels were measurable in age-matched and cachectic mice (data not shown). The commercial assays used were previously successful in measuring the cytokine response to lipopolysaccharide in mouse blood. Neither tissue levels of each cytokine nor mRNA levels were measured in this study.

Finally, because tumor growth is modified by the immune system (20, 50), we implanted the R-1 tumor into nude mice. Cachexia developed in the nude mice even though they lacked mature T lymphocytes. Body weights, blood glucose, epididymal fat pad weights, and muscle weights declined significantly in tumor-bearing nude mice, $P < 0.05$ vs. the nontumor-bearing control group. The cytokines IL-1β, IL-6, and TNF-α were also undetectable in cachetic nude mice. Tumor growth in nude mice was significantly greater than in CD2-F1 mice, $1.92 \pm 0.18$ vs. $1.25 \pm 0.13$ g, $P < 0.05$, respectively, on day 17.

### Drug Treatment of Cachexia

In previous studies with the C-26 tumor model (12, 52), treatment with indomethacin and dexamethasone was found to have modest protective effects. To determine whether these drugs have positive effects in this model and whether the effects mimic those already described, tumor-bearing mice were administered indomethacin and dexamethasone.

Indomethacin treatment of tumor-bearing mice. When indomethacin was administered at the beginning of weight loss (i.e., from day 12), there was a significant dose-related attenuation of weight loss (Table 2) and tissue losses (Table 3) compared with vehicle-treated tumor-bearing mice.

### Table 1. Tissue weights and serum concentrations of glucose in control mice and in R-1 tumor-bearing mice on day 17 posttumor implantation

<table>
<thead>
<tr>
<th>Group</th>
<th>EDL</th>
<th>Gastrocnemius</th>
<th>Soleus</th>
<th>Fat pad</th>
<th>Serum glucose</th>
<th>Tumor</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age-matched</td>
<td>12.2 ± 0.2</td>
<td>151 ± 2</td>
<td>7.1 ± 0.2</td>
<td>0.23 ± 0.04</td>
<td>322 ± 6</td>
<td>—</td>
</tr>
<tr>
<td>Day 10</td>
<td>12.5 ± 0.2</td>
<td>140 ± 4</td>
<td>6.7 ± 0.3</td>
<td>0.20 ± 0.02</td>
<td>239 ± 19</td>
<td>0.30 ± 0.08</td>
</tr>
<tr>
<td>Day 14</td>
<td>10.0 ± 0.3*</td>
<td>123 ± 3*</td>
<td>5.7 ± 0.3*</td>
<td>0.03 ± 0.01*</td>
<td>166 ± 30*</td>
<td>1.12 ± 0.04</td>
</tr>
<tr>
<td>Day 17</td>
<td>9.8 ± 0.3*</td>
<td>114 ± 4*</td>
<td>5.6 ± 0.4*</td>
<td>0.01 ± 0.00*</td>
<td>106 ± 6*</td>
<td>1.51 ± 0.12</td>
</tr>
</tbody>
</table>

*Values are means ± SE; n = 6 and 12 for control and tumor-bearing mice, respectively; no. in paren, no. of animals/group. EDL, extensor digitorum longus.

### Table 2. Body weights of control and R-1 tumor-bearing mice given indomethacin or dexamethasone

<table>
<thead>
<tr>
<th>Drug Treatment</th>
<th>Body Wt With Tumor Removed, g</th>
<th>%Initial</th>
<th>Body Wt With Tumor Removed, g</th>
<th>%Initial</th>
</tr>
</thead>
<tbody>
<tr>
<td>Indomethacin treatment, starting day 12</td>
<td>25.6 ± 0.4 (5)</td>
<td>106</td>
<td>24.9 ± 0.7 (5)</td>
<td>108</td>
</tr>
<tr>
<td>Indomethacin treatment, starting day 14</td>
<td>24.7 ± 0.7 (5)</td>
<td>107</td>
<td>24.1 ± 0.7 (5)</td>
<td>105</td>
</tr>
<tr>
<td>Dexamethasone treatment, starting day 12</td>
<td>17.3 ± 0.7 (6)</td>
<td>71</td>
<td>19.3 ± 0.3 (5)</td>
<td>84</td>
</tr>
<tr>
<td>Dexamethasone treatment, starting day 14</td>
<td>17.0 ± 0.7 (7)</td>
<td>77</td>
<td>18.7 ± 1.6 (5)</td>
<td>84</td>
</tr>
</tbody>
</table>

*Values are means ± SE; no. in paren, no. of animals; ND, not done. *$P < 0.05$ vs. vehicle-treated tumor-bearing mice.

Serum IL-1β, IL-6, and TNF-α were monitored, and no reliable levels were measurable in age-matched and cachectic mice (data not shown). The commercial assays used were previously successful in measuring the cytokine response to lipopolysaccharide in mouse blood. Neither tissue levels of each cytokine nor mRNA levels were measured in this study.
The lowest dose of indomethacin, 0.7 mg/kg, had no effect on body weight, hypoglycemia, or tissue weights. However, with a higher dose, 2.0 mg/kg, on day 18, the body weight (with the tumor removed) of the vehicle-treated group was 17.3 ± 0.7 g (71% of initial body weight), whereas the group given indomethacin was 19.7 ± 0.3 g (80% of initial, P < 0.05 vs. the vehicle-treated group). When the tumor was removed, the group receiving indomethacin at a dose of 6.0 mg/kg had body weights of 21.2 ± 4.4 g (93% of initial, P < 0.05 vs. the vehicle-treated group).

The gastrocnemius weights were significantly greater (P < 0.05) in the 2.0 and 6.0 mg/kg indomethacin-treated mice, 100 ± 3 and 102 ± 2 mg, respectively, compared with the vehicle-treated group (80 ± 2 mg). These muscle weights were, nevertheless, significantly less than those recorded in the healthy age-matched control group, P < 0.05.

Despite an effect of treatment on body and gastrocnemius muscle weights, circulating glucose concentrations and the epididymal fat pad weights were not different from the vehicle-treated tumor-bearing group (Table 3). When indomethacin (0.7–6.0 mg/kg) was administered beginning when weight loss was maximal (from day 14), there was no significant weight gain or attenuation of cachectic losses compared with vehicle-treated tumor-bearing mice (Table 3).

Interestingly, R-1 tumor size in indomethacin-treated mice became significantly larger than in vehicle-treated mice in both studies (Table 3).

Dexamethasone treatment of tumor-bearing mice. When dexamethasone was administered at the beginning of weight loss (from day 14), there was a significant dose-related attenuation of cachexia compared with vehicle-treated tumor-bearing mice (Tables 2 and 4). Specifically, although the lowest dose, 33 mg/kg, had no effects, when a higher dose was given, on day 18 posttumor implantation, the weight of the vehicle-treated group when the tumor was removed was 1.0 ± 0.7 g (84% of initial weight), whereas in the dexamethasone-treated group this was 19.8 ± 0.4 g (97% of initial), P < 0.05. The highest dose, 300 mg/kg, had effects equivalent to 100 mg/kg, i.e., 20.5 ± 0.5 g with tumor removed (101% of initial), also P < 0.05 vs. the vehicle-treated group.

Table 4. Muscle and adipose tissue weights and serum glucose concentrations in control mice and tumor-bearing mice given dexamethasone

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Serum Glucose, mg/dl</th>
<th>%Decline</th>
<th>Fat Pad, g</th>
<th>%Decline</th>
<th>Gastrocnemius, mg</th>
<th>%Decline</th>
<th>Tumor, g</th>
<th>%Increase</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Dexamethasone treatment, starting day 12</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age-matched</td>
<td>5</td>
<td>304 ± 9</td>
<td>—</td>
<td>—</td>
<td>124 ± 3</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Vehicle</td>
<td>7</td>
<td>161 ± 14</td>
<td>47</td>
<td>0.01 ± 0.00</td>
<td>95</td>
<td>79 ± 3</td>
<td>36</td>
<td>1.52 ± 0.07</td>
<td>—</td>
</tr>
<tr>
<td>100 mg/kg</td>
<td>10</td>
<td>246 ± 12*</td>
<td>19</td>
<td>0.07 ± 0.01*</td>
<td>63</td>
<td>86 ± 2*</td>
<td>31</td>
<td>1.74 ± 0.07</td>
<td>14</td>
</tr>
<tr>
<td>300 mg/kg</td>
<td>8</td>
<td>224 ± 10*</td>
<td>26</td>
<td>0.05 ± 0.01*</td>
<td>73</td>
<td>88 ± 1*</td>
<td>29</td>
<td>1.28 ± 0.09</td>
<td>16</td>
</tr>
<tr>
<td><strong>Dexamethasone treatment, starting day 14</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age-matched</td>
<td>5</td>
<td>240 ± 11</td>
<td>—</td>
<td>0.16 ± 0.02</td>
<td>—</td>
<td>122 ± 4</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Vehicle</td>
<td>5</td>
<td>144 ± 8</td>
<td>40</td>
<td>0.02 ± 0.01</td>
<td>88</td>
<td>87 ± 5</td>
<td>29</td>
<td>1.71 ± 0.22</td>
<td>—</td>
</tr>
<tr>
<td>33 mg/kg</td>
<td>3</td>
<td>142 ± 30</td>
<td>41</td>
<td>0.07 ± 0.04</td>
<td>56</td>
<td>91 ± 5</td>
<td>25</td>
<td>1.90 ± 0.21</td>
<td>11</td>
</tr>
<tr>
<td>100 mg/kg</td>
<td>3</td>
<td>187 ± 10*</td>
<td>22</td>
<td>0.06 ± 0.04</td>
<td>63</td>
<td>82 ± 4</td>
<td>33</td>
<td>1.95 ± 0.20</td>
<td>14</td>
</tr>
<tr>
<td>300 mg/kg</td>
<td>6</td>
<td>211 ± 10*</td>
<td>12</td>
<td>0.05 ± 0.01</td>
<td>69</td>
<td>85 ± 2</td>
<td>30</td>
<td>1.72 ± 0.26</td>
<td>0</td>
</tr>
</tbody>
</table>

Values are means ± SE. All tumor-bearing groups, P < 0.05 vs. control. All data from tumor-bearing mice were significantly less than control mice, P < 0.05. *P < 0.05 vs. vehicle-treated tumor-bearing mice.
In this study, circulating glucose concentrations, the epididymal fat pad weights, and the gastrocnemius weights were significantly higher in dexamethasone-treated mice ($P < 0.05$, Table 4). Despite protection against the cachectic losses, body and tissue weights remained less in the dexamethasone-treated mice than in the age-matched control group, $P < 0.05$.

When dexamethasone was administered beginning when weight loss was maximal (from day 14), there was no significant weight gain or attenuation of tissue losses compared with vehicle-treated tumor-bearing mice regardless of the dose (33–300 mg/kg, Table 2). However, there was a significant attenuation of hypoglycemia in the animals given this treatment regimen ($P < 0.05$ vs. the vehicle-treated tumor-bearing group). Dexamethasone treatment had no effect on tumor growth whether given before or after the induction of cachexia. Blood glucose, tissue weights, and tumor size are shown in Table 4.

Role of the Ubiquitin-Proteasome Pathway in Cachexia

Protein breakdown by incubated muscles. The amino acids measured, methionine, phenylalanine, and tyrosine, are neither synthesized nor metabolized by muscle. Therefore, net production of these amino acids can be used as a measure of protein breakdown. In the conditions described here for measuring amino acid production from incubated muscles, only proteolysis by the nonlysosomal and calcium-independent pathways of protein degradation was measured.

Although only data for tyrosine are shown, release of methionine and phenylalanine was similar. Net tyrosine production by incubated EDL muscles from cachectic animals was greater than in muscles from control mice ($P < 0.05$; Fig. 2A). Figure 2A shows that the elevated tyrosine release was suppressed by the specific proteasome inhibitor lactacystin (11). Although statistical significance was not achieved, there was a trend toward increasing the suppression of amino acid release by lactacystin as the cachexia became more severe (Fig. 2B).

Protein ubiquitination. With fraction II of the gastrocnemius muscle homogenate, ubiquitin conjugation to endogenous muscle proteins was measured and found to be greater in muscle extracts from cachectic mice than in those from control muscles ($P < 0.05$; Fig. 3A). This enhancement of ubiquitin conjugation could be due to greater activity of the pathway enzymes or greater susceptibility of muscle proteins in cachectic animals. In the same preparation, ubiquitin conjugation to model substrates of the N-end rule ubiquitination pathway, lysozyme and $\alpha$-lactalbumin, was also elevated in cachectic mice, compared with healthy controls (Figs. 3, B and C). Thus these observations with exogenous substrates indicate enhanced activity of the ubiquitin-proteasome pathway.

Biochemical parameters. Ubiquitinated proteins are targeted for degradation by the proteasome, and a larger proportion of muscle proteolysis is thought to occur in the N-end rule pathway (46). N-end rule selectivity is through the ubiquitin-protein ligase, E3a, and its cognate enzyme, $E2_{14k}$ (61). Intracellular levels of ubiquitin-protein conjugates and $E2_{14k}$ were compared in muscles from cachectic and age-matched controls.

In steady-state conditions, i.e., without any inhibitors of the proteasome or deubiquitination enzyme present, no elevation in ubiquitin-protein conjugate levels in gastrocnemius muscles from cachectic mice, relative to soluble protein content, was observed during severe cachexia on days 17 and 24 posttumor implantation. In these same muscles, $E2_{14k}$ protein was quantitated and also did not change. The lack of change in muscle $E2_{14k}$ protein was consistent with only nonsignificant changes in levels of $E2_{14k}$ mRNA (14 and 80% increases in 2 separate studies).

The 20S proteasome activity in gastrocnemius muscles from tumor-bearing mice did not appear to change from that in control animals (4.91 ± 1.08 vs. 3.51 ± 0.36 pmol 7-amino-4-methylcoumarin·s$^{-1}$·mg protein$^{-1}$, respectively).
DISCUSSION

In an effort to improve our knowledge of the mechanisms of cachexia, the murine R-1 tumor model of cancer cachexia was developed. Studies described here were performed with this newly identified tumor that clearly induces severe and reproducible cachexia. The pattern of cachexia included a loss of both muscle and adipose tissue and significant hypoglycemia. The tissue losses that developed in this model were comparable to other cachectic tumor models (3, 55). Weight loss started when the tumor was <1% of body weight. This small tumor size suggested that effects by this tumor were not due to a parasitic type of interaction, in which competition for nutrients between the tumor and host would cause a nutritional deficiency state in the mouse. The cachetic changes were all unrelated to food intake. Although total food intake declined briefly in the tumor-bearing mice described in this study, when intake is expressed on the basis of body weight, these animals actually ate more than control mice. The differences in food intake between control and cachetic mice were not statistically different and hence the data argue against a decline in food intake being a contributory factor to the cachexia. No further work in this regard was undertaken.

The contribution of cytokines to cachexia has been well studied (49, 63). In the C-26 tumor model, it is apparent that TNF-α and leukemia inhibitory factor do not contribute (63), IL-1 contributes within the tumor but not in peripheral tissues (49, 50), and IL-6 contributes to the cachexia in some, but not all, C-26 models (12). As described earlier with C-26 tumor-bearing mice (12), the pro-inflammatory cytokines IL-1β and TNF-α were both undetectable in the blood of R-1 tumor-bearing mice.

Although C-26 cells can secrete IL-6 in culture (12, 49), no such levels were found in R-1-conditioned medium (data not shown). Further, in earlier C-26 tumor studies that used a similar cytokine measurement assay, plasma IL-6 levels were significantly elevated, up to 1 mg/ml (12, 31, 43, 49). Blockade of IL-6 attenuated this cachexia (12, 38, 43). However, circulating levels of IL-6 did not become elevated in cachetic animals bearing the R-1 tumor. Blood IL-6 levels were undetectable even in mice where cachexia was severe.

The lack of IL-6 involvement in R-1-induced cachexia may partially explain why the drugs administered to R-1 tumor-bearing mice, indomethacin and dexamethasone, did not reproduce the results found in earlier work (12). These drugs have had limited effects in other experimental and clinical studies. For example, underweight cancer patients given either the glucocorticoid prednisolone or indomethacin suffered less pain and had longer survival times that the placebo group (28). Further, body weight and mid-arm muscle circumference were significantly greater in those patients who received prednisolone (28).

The administration of indomethacin had protective effects in several rodent models (8, 17, 34, 51), including a Lewis lung carcinoma made cachectic by transfection of IL-6 (35). Interfering with the prostaglandin pathway with dietary eicosanoids or acetylsalicylic acid was also successful in animal models of cachexia (8, 57), even when cytokines were not present as mediators of the cachexia (34), but this was not reproduced by administering indomethacin to mice implanted with the R-1 tumor.

Despite supportive experimental and clinical studies with these anti-inflammatory agents, neither dexamethasone nor indomethacin is part of the normal
therapy for cachectic cancer patients (19, 37). Because glucocorticoids cause protein loss (2, 39, 40, 42), chronic administration of this type of drug can, in fact, induce a cachectic-like state. Furthermore, endogenous glucocorticoid hypersecretion can have similar effects. However, in the C-26 model, the lack of protection by daily treatment with the glucocorticoid receptor antagonist, RU-38486 (data not shown), suggests that these hormones were not involved in the weight and tissue losses. The similar tissue losses between the treated and untreated groups when treatment was begun on day 14 showed that dexamethasone itself did not exacerbate the cachexia. On the other hand, nonsteroidal anti-inflammatory drugs such as indomethacin cause gastropathy (10), and this can deter the chronic use of such intervention. Furthermore, the stimulation of tumor growth by indomethacin that we and others (12) found would be detrimental if it occurred in a human cancer.

As noted in the R-1 model, dexamethasone and indomethacin were not effective when given in a clinically relevant schedule, i.e., during weight loss. Although some positive effects were noted in this report when they were given before weight loss, the mechanism(s) by which this occurred, being independent of IL-6, is uncertain.

A possible mechanism through which these drugs protected against tissue losses that developed in other models is by decreasing protein breakdown (2, 39, 40, 42). However, measurements of protein metabolism were not conducted in these current studies because the cachexia seen in the R-1 model was different (no detectable IL-6) from other models.

To elucidate the mechanisms that cause the severe muscle wasting of cachexia, cellular proteolytic pathways were investigated. Research has suggested that the ubiquitin-proteasome pathway is the major pathway of muscle protein loss in normal and cachectic states (2, 24, 25, 27, 44, 52, 56, 60). As an example, when rats were inoculated with the cachexia-inducing Yoshida AH-130 ascites tumor, the ubiquitin-proteasome pathway system of protein degradation was upregulated, as indicated by increased mRNA levels of ubiquitin and proteasome subunits (24, 53). Furthermore, muscle intracellular ubiquitin and ubiquitin-protein conjugates were elevated (24). Similar effects were found in muscles from cachetic mice bearing the C-26 tumor (13) and the MAC16 tumor (27).

In EDL muscles from cachetic mice, an increase in net protein loss was shown by the weight loss and the decrease in myofibrillar protein content. This was despite there being no change in endogenous soluble protein content. The increase in protein degradation was further demonstrated by showing a significantly greater production of amino acids by cachetic muscles, when compared with those from control animals. Exposure of incubated muscles to a proteasome inhibitor, lactacystin (11), identified the major pathway of elevated protein loss as the ubiquitin-proteasome pathway. When lactacystin was present, amino acid production by EDL muscles was suppressed from both cachetic and healthy muscles. As in cancer cachexia, a reduction in elevated proteolysis was accomplished by proteasome inhibitors in several pathological states (52). In the work reported here, the proteasome inhibitor suppressed amino acid release from cachetic EDL muscles to the same level as those from control mice. Significantly, lactacystin-sensitive proteolysis became greater as the tissue wasting became more severe. This cachexia-related proteasome-dependent increase in protein degradation was established conclusively that the ubiquitin-proteasome pathway has a central role in cachectic muscle loss caused by cancer.

The elevated proteolysis in cachetic muscles was corroborated by the activity of the ubiquitin-proteasome pathway, which also increased with R-1-induced cachexia. Ubiquitin conjugation of endogenous soluble proteins and N-end rule ubiquitination system proteins were increased in muscles of tumor-bearing mice. The N-end rule for protein degradation through the proteasome is of key importance in muscle tissue, possibly accounting for 50–80% of cellular soluble protein degradation (46). A recent report (44) showed that the activity of this pathway also increased in the Yoshida AH-130 model of cancer cachexia. These concurrent data underscore the importance of the ubiquitin-proteasome pathway in tumor-induced muscle wasting and focus attention on this pathway for future investigations.

By using gastrocnemius muscles removed from the same animals that provided the EDLs used in the studies described previously, specific components of the ubiquitin-proteasome pathway were measured, i.e., the intracellular ubiquitin-protein conjugates and the N-end rule specific E2 ubiquitin-conjugating enzyme E2\textsubscript{14k}. Muscle levels of ubiquitin-protein conjugates increased in other models of protein degradation (57, 60, 62), including cancer cachexia (13, 27, 57). It is expected that these elevations would be consistently present at all time points during cachectic muscle loss. However, because we did not find increases in this part of the ubiquitin-proteasome pathway at a time when cachexia was severe, whether it develops is not likely to be time dependent regarding tumor implantation or degree of cachexia and is not necessarily required for the muscle wasting to occur. Increased levels of the mRNA for E2\textsubscript{14k} were found in muscles from fasted rats (60), as well as in cancer cachexia (27, 52). We also found an increase in E2\textsubscript{14k} mRNA in one study. Still, this was not consistent, and the lack of any increase in muscle levels of the E2\textsubscript{14k} protein suggest that, like ubiquitin-protein conjugates, it is not a limiting factor in muscle wasting. Other components of this pathway, such as proteasome subunits, the mRNAs of which increased in cancer cachexia (13, 24, 27, 52), may be more important in determining the rate of activity. On the other hand, additional mechanisms that can be used to determine the contribution of the ubiquitin-proteasome pathway to muscle loss in cachectic states may be present, for example, the regulation of activity of components of the ubiquitin-proteasome pathway by phosphorylation or the availability of protein to this pathway through phosphorylation or the availability of protein to this pathway through...
separation of components of the myofibrillar complex (30, 45).

The distinction between previous findings and those reported here may result from the contribution of model-specific mediators. The proinflammatory cytokine TNF-α contributes to AH-130-induced muscle losses (7, 54) and IL-6 contributes to C-26-related muscle losses (48, 63). Because both of these cytokines upregulate the ubiquitin-proteasome pathway (15, 16, 59), the increases in ubiquitin-proteasome pathway components shown in the AH-130 and parent C-26 tumor models of cachexia may represent cytokine-induced changes that are associated with, but are not required for, the muscle losses. Because neither TNF-α nor IL-6 contributes to R-1 cachexia, the ubiquitin-protein conjugates and E2$_{4k}$ mRNA were not upregulated in cachetic muscles from mice bearing the cytokine (non-IL-1β, IL-6, and TNF-α)-independent R-1 tumor. Although an increase in E2$_{4k}$ mRNA was found in cachetic mice bearing the cytokine (non-IL-6 and TNF-α)-independent MAC16 tumor, these data were not confirmed by measurement of intracellular protein levels of this E2 (27).

In conclusion, we report here that the ubiquitin-proteasome pathway is an important part of the mechanism of protein wasting in cancer cachexia. The clinical impact that cachexia has for the cancer patient makes it necessary to develop efficacious treatments for this pathology. As we increase our understanding of cachexia, new targets will appear against which therapies can be developed. The increased contribution of the ubiquitin-proteasome system to muscle proteolysis suggests that it would be an excellent target for therapeutic intervention.

Address for reprint requests and other correspondence: P. J. Elliott, ProScript, 38 Sidney St., Cambridge, MA 02139 (E-mail: pelliott@proscript.com).

Received 25 February 1999; accepted in final form 22 April 1999.

REFERENCES


