Increased Gene Expression of Brown Fat Uncoupling Protein (UCP)1 and Skeletal Muscle UCP2 and UCP3 in MAC16-induced Cancer Cachexia

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INTRODUCTION

Cancer cachexia is a clinical syndrome of profound weight loss, anorexia and weakness, which occurs in many forms of malignancy (1). Cachexia is a major contributor to morbidity and premature death in cancer patients; it affects over two-thirds of patients with advanced cancer and, in most cancer types, survival is inversely correlated with the weight loss (2, 3). Unfortunately, treatment of this common and debilitating condition remains unsatisfactory, largely because the fundamental mechanisms that underlie weight loss are not fully understood.

Weight loss is not simply caused by increased nutrient consumption by the tumor itself, because the tumor burden is usually trivial (<5% of total body weight), but rather by concerted changes in host metabolism induced by the tumor or the host’s response to it (4). These changes include hypophagia, increased energy expenditure, and depletion of fat and muscle (4–7). The failure to maintain or increase energy intake to compensate for energy loss is a major factor in causing cachexia (8, 9). Various tumor products that inhibit feeding and/or stimulate energy expenditure have been identified (1), but the specific molecular mechanisms responsible are not known.

In this study, we focused on the possible roles of the UCPs (1, 2, and 3), which are postulated to be involved in the control of energy metabolism. UCP1, a mitochondrial protein expressed exclusively in BAT, is responsible for dissipating energy as heat instead of generating ATP from the oxidation of FFA (10); it determines the thermogenic capacity of BAT, a major heat-producing tissue in rodents and human neonates (11). Gene expression of UCP1 is stimulated by the sympathetic nervous system, mediated by the β3 adrenoreceptor (12–15).

Recently, two structurally similar proteins with high homology to UCP1 have been identified and designated UCP2 and UCP3 (16, 17). UCP2 is widely expressed in most tissues including white fat, BAT, muscle, heart, and liver, whereas UCP3 is mainly confined to thermogenic tissues such as skeletal muscle and brown fat in rodents; in humans, it is predominantly expressed in muscle (16–18). It has been suggested that these novel proteins also uncouple mitochondrial oxidative phosphorylation and contribute to thermogenesis in vivo (19, 20). This could be important for energy expenditure in humans, who virtually have no BAT after the neonatal period (12). However, UCP3 expression in muscle is up-regulated by fasting, a condition in which there are substantial decreases in BAT thermogenic activity (12, 21). Other possible physiological functions of UCP2 and UCP3 have been suggested, for example, involvement in the use of FFA (22); high levels of FFA are postulated to be responsible for the up-regulation of muscle UCP3 (23).

The possible roles of the UCPs in the increased lipid catabolism and/or the enhanced thermogenic activity found in cancer cachexia have not been systematically investigated and may vary among different cancers and species. Little is known about the changes of BAT UCP1 mRNA in cancer cachexia, although increased skeletal muscle UCP2 and -3 mRNA has recently been reported in a rat cancer cachexia model that displays increased energy expenditure (24).

Here, we studied the murine colonic adenocarcinoma, MAC16, a model for many human gastrointestinal and pancreatic cancers (25). The MAC16 induces profound weight loss (up to 30%) with a minimal tumor burden (<3%) and prevents the compensatory hyperphagia that normally follows weight loss (25, 4). Changes in energy expenditure or thermogenic capacity have not been characterized in MAC16-bearing mice. Recently, circulating catabolic factors PIF and LMF that, respectively, cause proteolysis (26) and lipolysis (27) have been isolated from MAC16-tumor mice (26). In vitro, LMF enhances FFA release from adipose tissue (4), and, in vivo, it specifically depletes carcass lipid, increases serum glycerol levels, and stimulates oxygen uptake by BAT (28). Both PIF and LMF are also found in the urine of patients with cancer cachexia.

The aim of this study was to determine whether the expression of UCP1, -2, and -3 was altered in mice bearing the MAC-16 tumor and/or the enhanced thermogenic activity found in cancer cachexia.

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The abbreviations used are: UCP, uncoupling protein; BAT, brown adipose tissue; LMF, lipid-mobilizing factor; PIF, proteolysis-inducing factor; FFAs, free fatty acid(s); TNF, tumor necrosis factor; IL, interleukin; GDP, guanosine diphosphate.
to relate any such alterations to changes in food intake, body weight, and composition. UCP1, -2, and -3 mRNA levels were measured in BAT and gastrocnemius muscle using Tri-reagent (Sigma; Poole, United Kingdom) and stored at −80°C. Muscle, interscapular BAT, and gonadal fat pads were dissected, snap-frozen in liquid nitrogen, and stored at −80°C. The tumor was also dissected out and weighed. Plasma TNF-α, IL-1β, and IL-6 were all analyzed with ELISA kits (Crystal Chem; Chicago, IL). Plasma FFA levels were measured using an enzymatic colorimetric assay kit (Boehringer Mannheim, Mannheim, Germany).

Expression of UCP1, -2, and -3 mRNA. Total RNA was extracted from BAT and gastrocnemius muscle using TRI-reagent (Sigma; Poole, United Kingdom) and RNA concentration determined from the absorbance at 260 nm. Twenty μg of total RNA per sample was applied to a 1% agarose-formaldehyde gel and separated by electrophoresis. RNA was transferred overnight to a charged membrane by capillary blotting and then cross-linked under UV light.

UCP1, -2, and -3 mRNAs were detected by Northern blotting in conjunction with the chemiluminescence method. The membranes were prehybridized in Easyhyb solution (Boehringer Mannheim) at 42°C for 1 h and were hybridized in the same solution with a digoxigenin-labeled 32-mer antisense oligonucleotide probe for mouse UCP1 (33), or digoxigenin-labeled 30-mer oligo probes for mouse UCP2 (5′-ACTGTTTGACAGTAGGTG CAGAGCCTACGATCTTTGCTCC-3′; GenBank accession number: U96135) and UCP3 (5′-CGTAGCAGACACATGACGACACTTGC-3′; GenBank accession number: AB008216), respectively. Each blot was stripped and reprobed for 18S rRNA with a 31-mer digoxigenin-labeled oligonucleotide, as described previously (34). The amount of mRNA was expressed as the ratio of UCP mRNA:18S rRNA signals.

Statistical Analyses. Data are expressed as means ± SE. Differences in food intake and body weight between groups were compared using two-way ANOVA coupled to a Bonferroni t test. Tissue weights, plasma levels of FFA and cytokines, and mRNA levels of the UCPs were compared by one-way ANOVA. ARCUS statistical software (Medical Computing, Aughton, United Kingdom) was used throughout. A P of 0.05 or less was considered significant.

RESULTS

Food intake was similar in the freely fed control and MAC16-tumor-bearing groups until day 8 after tumor inoculation; thereafter, MAC16 mice ate generally less compared with controls, with a progressive decrease after day 15 and a precipitous 60% reduction on day 18 (Fig. 1a and b). Total food intake between days 1 and 18 was 14% less in the MAC16 group than in controls (P < 0.01; Table 1).

Body weight did not show apparent separation between MAC16 and freely fed animals until day 13 after tumor transplantation (Fig. 2). Thereafter, non-tumor-bearing freely fed controls continued to gain weight, whereas tumor-bearing mice weighed progressively less; on day 18, the final body weight was 24% (P < 0.01) lower than that of freely fed animals (Table 1). Tumor weight was 0.38 ± 0.03 g, averaging 1.8% of final body weight, and was inversely correlated with final body weight (r = −0.56; P < 0.02). Pair-fed animals also lost weight after day 13 but to a lesser degree than the MAC16 mice (Fig. 2); final body weight was 10% (P < 0.01) lower than in the freely fed group and 16% higher than in the tumor-bearing group (P < 0.01; Table 1).

There was severe loss of gonadal fat in the MAC16 group (−67%; P < 0.01) and a lesser reduction in the pair-fed group (−21%; P < 0.01), compared with freely fed controls (Table 1). Intercapular BAT also weighed less in both MAC16 and pair-fed groups (−36%...
UCPs AND TUMOR-INDUCED CACHEXIA

Table 1 Food intake, body weight, fat, muscle weight, UCP2 and UCP3 mRNA levels in female NMRI mice

<table>
<thead>
<tr>
<th>Non-tumor-bearing</th>
<th>Freely fed</th>
<th>Pair-fed</th>
<th>MAC16-bearing</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>16</td>
<td>16</td>
<td>16</td>
</tr>
<tr>
<td>Total food intake (g)</td>
<td>84.8 ± 2.2</td>
<td>72.6 ± 1.2**</td>
<td>72.6 ± 1.2**</td>
</tr>
<tr>
<td>Body weight (g)</td>
<td>23.7 ± 0.3</td>
<td>23.7 ± 0.3</td>
<td>24.4 ± 0.3</td>
</tr>
<tr>
<td>Initial (g)</td>
<td>27.7 ± 0.7</td>
<td>24.9 ± 0.4**</td>
<td>21.0 ± 0.6**††</td>
</tr>
<tr>
<td>Final (g)</td>
<td>+17 ± 0.8</td>
<td>+5.1 ± 0.3**</td>
<td>−14 ± 0.7**††</td>
</tr>
<tr>
<td>Weight change (%)</td>
<td>63%</td>
<td>24%</td>
<td>70%</td>
</tr>
<tr>
<td>Gonadal fat mass (mg)</td>
<td>232 ± 14</td>
<td>183 ± 17**</td>
<td>77 ± 8**††</td>
</tr>
<tr>
<td>Interscapular BAT</td>
<td>111 ± 5</td>
<td>89 ± 7**</td>
<td>74 ± 8**</td>
</tr>
<tr>
<td>Weight (mg)</td>
<td>1.40 ± 0.35</td>
<td>1.35 ± 0.31</td>
<td>1.01 ± 0.26</td>
</tr>
<tr>
<td>BAT UCP2 mRNA (ratio)</td>
<td>1.88 ± 0.34</td>
<td>2.15 ± 0.53</td>
<td>1.71 ± 0.48</td>
</tr>
<tr>
<td>BAT UCP3 mRNA (ratio)</td>
<td>142 ± 5</td>
<td>132 ± 6</td>
<td>114 ± 4**</td>
</tr>
</tbody>
</table>

† Data are mean ± SE. n = 8–10 each group for BAT UCP2 and UCP3 measurement. †† Statistical significance of differences between groups: ** P < 0.01 versus freely fed controls; † P < 0.05 versus pair-fed group.

and −24%, respectively, both P < 0.01 versus freely fed controls). Gastrocnemius muscle weight showed a similar relationship, with decreases below controls of 20% (P < 0.01) in MAC16 and 7% (P < 0.05) in pair-fed groups (Table 1).

As shown in Table 2, plasma leptin levels fell significantly in tumor-bearing mice (−87%, P < 0.01) and to a lesser degree in the pair-fed group (−70%; P < 0.01). Plasma leptin positively correlated with both body weight (r, 0.80; P < 0.001; Fig. 3) and gonadal fat mass (r, 0.76; P < 0.001), across all of the three experimental groups. Plasma FFA concentrations were significantly higher in both MAC16 (+79%; P < 0.05) and pair-fed (+99%; P < 0.01) groups than in freely fed controls, with no significant difference between the two experimental groups (Table 2). Circulating concentrations of TNF-α, IL-1β, and IL-6 did not differ significantly between any of the three groups (Table 2).

In BAT, MAC16-tumor-bearing mice showed significantly increased UCP1 mRNA levels above freely fed controls (+63%; P < 0.01), whereas UCP1 mRNA levels were not changed in pair-fed animals compared with controls (Fig. 4). Neither UCP2 nor UCP3 expression in BAT was altered in MAC16-tumor-bearing or in pair-fed mice, relative to controls (Table 1).

In skeletal muscle, UCP2 mRNA levels were higher than in freely fed controls in both MAC16 mice (+183%; P < 0.05) and pair-fed animals (+130%; P < 0.05), with no significant difference between the experimental groups (Fig. 5). UCP3 mRNA levels in muscle showed significant increases above controls in MAC16 mice (+163%; P < 0.05) and in pair-fed animals (+253%; P < 0.01), again with no significant difference between these two groups (Fig. 6). There was a highly significant correlation between muscle UCP3 mRNA and plasma FFA levels (r, 0.70; P < 0.001; Fig. 7), but not between UCP2 mRNA and plasma FFA (r, 0.38; P > 0.05). Muscle UCP3 mRNA was also inversely correlated with gonadal fat mass (r, −0.43; P < 0.05), whereas UCP2 was not (r, −0.28; P > 0.05).

Core temperature was significantly lower in the MAC16 group than in the freely fed control group (36.1 ± 0.1 versus 38.5 ± 0.3; P < 0.01) at day 18 after tumor inoculation, whereas pair-feeding did not affect core temperature (38.0 ± 0.2) compared with freely fed animals (P > 0.05).

**DISCUSSION**

Consistent with the previous reports (25), mice bearing the MAC16 adenocarcinoma displayed cachexia, with progressive weight loss from 13 days after tumor inoculation and a 24% lower final weight than non-tumor-bearing controls. Weight loss seems to be attributable to tumor-related products, because final body weight was negatively correlated with tumor mass although tumor burden only accounted for <2% of body weight. The marked reductions in both white and brown fat and the moderate decrease in skeletal muscle mass are consistent with potent lipolytic and proteolytic effects of known products of this tumor, LMF and PIF (26). Despite severe wasting, mice bearing the MAC16 tumor did not show a compensatory increase in appetite, and, instead, total food intake was decreased, especially during the last few days. Thus, tumor products may also suppress feeding.

In pair-fed animals that consumed the same amount of food as the tumor-bearing animals, body weight was also reduced below controls.

**Table 2 Plasma concentrations of leptin, FFA, and cytokines in female NMRI mice**

<table>
<thead>
<tr>
<th>Non-tumor-bearing</th>
<th>Freely fed</th>
<th>Pair-fed</th>
<th>MAC16-bearing</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>11</td>
<td>11</td>
<td>11</td>
</tr>
<tr>
<td>Leptin (ng/ml)</td>
<td>2.3 ± 0.4</td>
<td>0.7 ± 1.0*</td>
<td>0.3 ± 0.4*††</td>
</tr>
<tr>
<td>FFA (μmol/ml)</td>
<td>206 ± 51</td>
<td>411 ± 42**</td>
<td>361 ± 57*</td>
</tr>
<tr>
<td>TNF-α (ng/ml)</td>
<td>3.4 ± 0.3</td>
<td>2.1 ± 0.2</td>
<td>3.0 ± 0.2</td>
</tr>
<tr>
<td>IL-1β (pg/ml)</td>
<td>117 ± 9</td>
<td>130 ± 10</td>
<td>15.1 ± 1.6</td>
</tr>
<tr>
<td>IL-6 (pg/ml)</td>
<td>17.7 ± 1.8</td>
<td>15.7 ± 3.5</td>
<td></td>
</tr>
</tbody>
</table>

† Data are mean ± SE. †† Statistical significance of differences between groups: * P < 0.05; ** P < 0.01 versus freely fed controls; †† P < 0.01 versus pair-fed group.

**Fig. 3. Correlation between plasma leptin levels and body weight in MAC16-tumor-bearing, pair-fed, and freely fed non-tumor-bearing mice. r = 0.80; P < 0.001; n = 33.**
but to a lesser degree, as were white and BAT weights; however, gastrocnemius muscle mass was not significantly affected. The significantly greater reductions in body fat and body weight in tumor-bearing than in pair-fed animals may suggest the importance of the lipolytic factor LMF in vivo (26). Our observations further highlight the complexity of cachexia in this model: it is not caused solely by reduced energy intake, and tissue breakdown and/or increased energy expenditure must contribute (see Fig. 2). Increased energy expenditure has been reported both in humans and in experimental animals with cancer cachexia (9, 35, 36, ), but the underlying molecular mechanisms are not fully identified.

Here, BAT UCP1 mRNA was increased in tumor-bearing mice, which indicated activation of non-shivering thermogenesis. This was a highly selective change, because UCP2 and -3 mRNA in the same tissue were unaltered. By contrast, the pair-fed animals showed no elevation in UCP1 mRNA, which suggested that the additional weight loss in tumor-bearing animals is partly due to inappropriately increased energy expenditure by BAT and/or to excessive catabolism, both presumably driven by tumor products. Non-shivering thermogenesis in BAT contributes substantially to resting energy expenditure in rodents (37, 38). Increased thermogenic activity of BAT as assessed by GDP binding has also been reported in several rat models of malignancy, such as leukemia and the Yoshida sarcoma (9, 39), and is likely to be sympathetically mediated stimulation as it is attenuated by propranolol treatment (9). Moreover, BAT oxygen uptake in vivo
is stimulated by a LMF, isolated from cancer patients, which showed similar lipidic effects to the MAC16-derived LMF (28). In our study, tumor-bearing mice had significantly lower body temperature at day 18, whereas pair-feeding did not alter body temperature, which suggested that MAC16-tumor products may independently induce hypothermia that can override additional heat produced by BAT activation. Separate studies from our laboratories have shown that PIF given peripherally can lower body temperature in normal mice. The mechanism of this effect is not known but may be attributable to an action on thermoregulatory centers in the brain or to loss of skeletal muscle protein. In addition, body temperature can decrease at a late stage of cachexia in leukemic rats (9) and in terminal cancer patients, which might be the results of emaciation. Structural alterations have been reported in BAT mitochondria from cancer cachectic mice, alterations that are similar to those cold-adapted animals and that are not seen in pair-fed animals (36). However, it is not clear whether the increased BAT thermogenic activity in MAC16 mice (a) reflects a compensatory mechanism in response to hypothermia induced by tumor product as occurs in cold-exposure (12, 40); or (b) results from a direct or indirect stimulatory effect of LMF on BAT.

The newly described UCP2 and UCP3 have been implicated in energy metabolism in skeletal muscle, an important site for thermogenesis, particularly in humans (16, 19). Their roles in cachectic states such as malignancy or infection remain unclear, although increased UCP2 and UCP3 expression in skeletal muscle was reported in rats bearing the Yoshida AH-130 ascites hepatoma (24), while our studies were in progress. In MAC16-tumor-bearing mice, gastrocnemius muscle UCP2 and-3 expression were markedly up-regulated, which is in agreement with the study by Sanchis et al. (24). Because skeletal muscle contributes to thermogenic response to cold (38), up-regulation of UCP homologous by the tumor could point to enhanced energy production that could exacerbate tissue losses. However, this notion is challenged by the fact that pair-feeding induced similar increases in muscle UCP2 and -3 expression. Muscle UCP2 and-3 may, therefore, serve other nonthermogenic functions, perhaps in lipid utilization (22, 23).

FFA are raised in some patients with cancer cachexia (41, 42), and, in our study, plasma FFA levels were comparably increased in both pair-fed and MAC16-tumor-bearing animals. Interestingly, we found a highly significant positive correlation between plasma FFA and muscle UCP3 mRNA, consistent with suggestions that FFA induce muscle UCP3 expression both in vivo (22, 43) and in vitro (23). Several studies demonstrate that skeletal muscle UCP3 mRNA levels are increased dramatically during fasting, which also raises circulating FFA levels (21, 22, 44). Because fasting reduces energy expenditure, increased muscle UCP3 expression is unlikely to regulate thermogenesis in this state but may instead play a role in the utilization of FFA or perhaps the removal of toxic free radicals resulting from enhanced β-oxidation of FFA. The inverse correlation between muscle UCP3 and fat mass observed in our study further supports the possible involvement of this protein in lipid mobilization and metabolism. Any direct association of muscle UCP2 to MAC16 was not established in this study.

Leptin induces weight loss by suppressing appetite and stimulating heat production, mobilizing fat with little loss of lean body mass (45). Intracerebroventricular leptin infusion increases BAT UCP1 mRNA, and UCP2 and UCP3 mRNA in brown and white adipose tissues as well as in skeletal muscle (30). In MAC16-tumor-bearing mice, circulating leptin fell markedly (~87%) in proportion to fat and weight loss, which suggests that leptin production has been severely suppressed in this model; this indicates that leptin is unlikely to mediate hypophagia, wasting, or enhanced UCP expression in MAC16-induced cancer cachexia. Indeed, low leptin levels would be predicted to stimulate hunger and feeding, which highlights the powerful appetite-suppressing effect of tumor products. Low or undetectable circulating leptin concentrations have also been reported in patients with lung cancer and weight loss (46) and gastrointestinal cancer (47). The unchanged levels of circulating TNFα, IL-1α, and IL-6 levels in MAC16-bearing mice make it unlikely that wasting or UCP expression are mediated by these cytokines. We are currently investigating whether UCP expression is affected by the MAC16 products, PIF and LMF.

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