Evidence for a Novel Serum Factor Distinct From Zinc Alpha-2 Glycoprotein That Promotes Body Fat Loss Early in the Development of Cachexia

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We provide evidence that a factor other than the previously identified lipid mobilizing factor, zinc alpha-2 glycoprotein, promotes lipolysis in the MCA-induced sarcoma-bearing cachexia model. Cachexia is characterized by progressive loss of adipose tissue and skeletal muscle without a concurrent increase in food intake to restore lost tissue stores. We compared tumor-bearing ad lib fed (TB) animals to nontumor bearing ad lib fed (NTB) animals or nontumor-bearing pair-fed (PF) animals at various time points throughout development of tumor derived cachexia. Prior to cachexia, the TB animals lost more than 10 ± 0.7% of their body fat before losing protein mass and decreasing their food intake. Fat loss occurred because adipocyte size, not number, was reduced. Increased turnover of palmitate and significantly higher serum triglyceride levels prior to cachexia were further indicators of an early loss of lipid from the adipocytes. Yet, circulating levels of norepinephrine, epinephrine, TNF-α, and zinc α-2 glycoprotein were not increased prior to the loss of fat mass. We provide evidence for a serum factor(s), other than zinc alpha-2 glycoprotein, that stimulates release of glycerol from 3T3-L1 adipocytes and promotes the loss of stored adipose lipid prior to the loss of lean body mass in this model.

INTRODUCTION

Several decades ago, DeWys and colleagues (1,2) published their seminal papers showing weight loss was common among cancer patients and that that weight loss significantly impacted prognosis. This weight loss, termed cachexia for the Greek word meaning bad condition, includes both lean and fat body mass. Although there is no concise definition for this condition (3), generally a cancer patient is considered cachectic if they have unintentionally lost more than 10% of their usual body weight in a year without concurrent anorexia (4).

Are lean and fat mass lost simultaneously or is one lost before the other? Several studies, have reported on body compositional changes in human cancer patients using a variety of techniques including anthropometry (5–7); whole body potassium determination (8–11); neutron activation (12,13); computerized tomography (14); bioelectric impedance (15,16); and the gold standard, dual-energy, x-ray absorptiometry (DXA or DEXA) (5,17–20). Most studies have been cross-sectional, but a few were longitudinal. None of the studies have followed the cancer patient before and after the onset of cachexia. Despite the limitations of study designs and measurement techniques, most studies have indicated body fat declines first when a patient suffers from a progressive disease (6,21).

A recent study evaluated time course of changes in body composition, measured using DEXA, in cancer patients who...
received palliative care (systemic anti-inflammatory treatment, recombinant erythropoietin treatment, and/or specialized nutritional support care) during a 4- to 62-mo follow-up of their disease (22). At the time of admission to the study, on average, the cohort had already lost 8 ± 9% of their body weight, and no effective tumor treatment was available. During the follow-up period, further loss of body fat mass was observed and not lean body mass, which was maintained or increased slightly. Interestingly, the amount of whole body fat was also a significant predictor of survival.

Halabi et al. (23) investigated the correlation between body mass index (BMI) and clinical outcome of patients with metastatic, castration-recurrent prostate cancer. A BMI greater than 25 (defined as overweight by the Centers for Disease Control and Prevention) had a protective effect against overall mortality and prostate cancer-specific mortality. These patients lived an average 5 mo longer.

Our knowledge and understanding of adipocyte biology and function has changed dramatically in the last decade. Adipose tissue is no longer considered just an energy storehouse but a dynamic endocrine organ that controls a complex network of important biological functions (24). These include energy homeostasis, immune function, cell signaling, and reproduction.

We propose that body fat loss occurs early, before loss of lean body mass and before onset of cachexia is clinically observed. Ideally, we would like to test this hypothesis in humans; however, ethical reasons make this difficult, so we have used an animal tumor-bearing model. We selected the methylcholanthrene (MCA)-induced sarcoma model. The tumor does not metastasize and has been used in more than 100 published studies including the original parabiosis studies (25), which demonstrated blood from a tumor-bearing animal promotes weight loss in the parabiosed nontumor-bearing pair.

The precise mechanism behind the loss of body tissue in the tumor-bearing host is still unknown. Our working hypothesis is malignant growth promotes changes in circulating factors that alter adipocyte biology prior to the clinical manifestations of cachexia. A number of hormones (including insulin, glucocorticoids [corticosterone for the rat], thyroid stimulating hormone, ACTH, norepinephrine and epinephrine), natriuretic peptides, and cytokines are known to affect lipolysis. The circulating glycoprotein, zinc alpha-2 glycoprotein (ZAG), has lipolytic activity (26,27). The specific aims of this study were twofold: 1) to determine if fat mass is lost prior to lean body mass and if that loss occurs prior to the clinical manifestation of cachexia; and 2) to determine if known circulating lipolytic factors are involved in promoting this early fat loss. This study is unique because we followed changes before and after the onset of cachexia. We report here that an unidentified serum factor, most likely a protein, distinct from zinc alpha-2 glycoprotein, promotes loss of lipid stored in adipocytes.

MATERIALS AND METHODS

Animals and Diets

Male Fischer 344 rats, ranging in weight from 240 to 300 g, were purchased from Charles River (Wilmington, MA) and housed in the vivarium at Pennington Biomedical Research Center (Baton Rouge, LA) or the University of North Carolina at Charlotte. This protocol was approved by the Institutional Animal Care and Use Committee at Pennington Biomedical Research Center and the University of North Carolina at Charlotte.

All animals were acclimated to individual housing for 7 to 10 days prior to the start of any experiment. Then each rat was weighed and randomly assigned to one of the following ad libitum fed groups: tumor-bearing (TB) or nontumor-bearing (NTB). The remaining animals were weight-matched to a tumor-bearing animal. Weight-matching was important to avoid pairing a tumor-bearing animal with a pair-fed control that weighed more. This group, called pair fed (PF), received the same amount of food that their matched tumor-bearing pair consumed the previous 24 h. Pair feeding started on Day 10 since food intake did not differ between the TB and NTB until after this day. All animals were fed rat chow (Purina; St. Louis, MO). The PF animals were fed twice a day, around 8 AM and 5 PM, to prevent gorging. Food intake and body weight were recorded daily.

On Day 1, the animals were anesthetized with isoflurane and the MCA-induced sarcoma implanted subcutaneously in the hind flank of the TB animals. The NTB animals, both ad libitum fed and pair-fed, were sham operated. Briefly, the hind flank was shaved and disinfected. A small skin piercing incision was made and using blunt surgical technique, a short tunnel was made under the skin. For the TB animals, a 2 mm³ piece of the tumor was placed in the tunnel whereas nothing was placed for the sham-operated animals. Then the wound was closed with wound clips.

At specific time points after tumor implantation, animals from the 3 groups were sacrificed. On Day 10, animals from the TB and NTB groups were sacrificed. On Day 15 and 21, animals from all 3 groups—TB, NTB, and PF—were sacrificed. All food was removed the evening prior to the day of sacrifice. This experiment has been repeated 3 times with similar results each time.

Tumor dimensions (length, width, height) were measured daily by the same investigator starting on Day 10 when the tumor was first palpable. From this measurement, the daily cubic volume was calculated. At the time of sacrifice, the tumor was carefully excised and weighed. Daily tumor weight was calculated from the ratio of the daily cubic volume to the final cubic volume as described by Morrison (28).

Analysis of Body Composition

Protein and fat content of Day 15 and Day 21 TB and PF animal’s carcasses were determined by chemical analysis. The
PF group, and not the NTB group, was included to account for differences as a result of changes in food intake. For the TB animals, the tumor was removed and its fat and protein content determined separately from the host’s carcass. Otherwise, the carcass comprised the whole body of the animal except for approximately 10 ml of blood and a 1 gram sample of subcutaneous, omental, and epididymal fat.

A known amount of water was combined with the animal’s carcass and then homogenized. A weighed aliquot of the homogenate was completely dried at 70°C. Body water content was calculated as the difference in weight before and after drying minus the portion of water added. The fat content of the sample was determined using the solvent extraction technique, a Soxhlet apparatus, and ethyl ether as the solvent. The remaining defatted sample was finely ground into a highly homogenous powder and the carcass protein content determined by the micro-Kjeldahl method (29). The percentage of fat and protein was calculated in relation to carcass mass.

Adipose Cell Size and Count

Adipocyte size and number were determined for a subcutaneous fat sample. Fat cells were fixed with osmium and then counted on a Beckman Multisizer III Coulter Counter (Beckman Coulter, Fullerton, CA).

Energy Expenditure

Daily energy expenditure was measured using the Oxymax system (Columbus Instruments, Columbus, OH). The animals were acclimated to individual housing and metabolic cage for at least 1 wk prior to implanting the tumor. The Oxymax system was kept closed except for the brief time each day the animal and its food were weighed. Pair-feeding started 10 days after tumor implantation.

Free Fatty Acid Turnover

Free fatty acid turnover was quantified using a primed, continuous infusion of 1–14C palmitate solubilized with albumin. On Day 17, 10 animals in the TB and NTB group were anesthetized (pentobarbital, 6 mg/100 g body weight) and catheters surgically implanted in the jugular vein and the carotid artery. A priming dose of 1–14C palmitate was slowly administered into the jugular catheter. This was followed by a 2.5 h continuous infusion (0.09 μCi 14C palmitate/min). Between 2 and 2.5 h after the start of the infusion, a blood sample was taken and the serum separated and stored at –80°C for analysis later.

Total free fatty acids were quantified using a modified method described by Falholt et al. (30). The amount of 14C labeling present in palmitate was quantified after the lipids were extracted from plasma (30) and further separated by thin layer chromatography. The plate was stained with Rhodamine-B dye and the palmitate region scraped into a liquid scintillation vial filled with liquid scintillation cocktail. Radioactivity (cpm) was measured by a scintillation counter. The samples were counted in triplicate and then averaged and corrected for counting efficiency (dpm).

Host free fatty acid turnover was calculated using the following equation:

\[
\text{infusion rate (dpm/min)} \times \frac{\text{specific activity (dpm/μmol)}}{\text{specific activity (dpm/ml)}}
\]

where the infusion rate was calculated by multiplying the infused activity (dpm/ml) by the rate of infusion (ml/min). The specific activity (dpm/μmol) was calculated by dividing the palmitate activity of the plasma sample (dpm/ml) by the total plasma free fatty acid concentration (μmol/ml). Turnover was normalized to the weight of the animal (kg).

Blood Collection and Analyses

At specific time points after tumor implantation, animals from each group were fasted overnight and then anesthetized and blood collected by cardiac puncture and appropriately processed for the collection of serum or plasma. Blood for quantitation of catecholamines (epinephrine and norepinephrine) was collected following decapitation.

Serum triglycerides were quantified enzymatically (TR0100; Sigma Aldrich, St. Louis, MO). Insulin (sensitive rat insulin RIA, SRI-13K), glucagon (GL-32K), and leptin (RL-83K) were quantified using kits purchased from Linco Research (Millipore, Billerica, MA). Adiponectin (44-ADPRT-E01) and ACTH (021-SDX018) were quantified using ELISA kits purchased from ALPCO Diagnostics (Windham, NH). Corticosterone was quantified using an RIA specific for rats purchased from ICN Biomedicals, Inc. (07–120102, Costa Mesa, CA). The rat cytokines, rIL-1β, rIL-6, rIL-10, rGRO/KC and rrTNF-α were quantified using a multiplex kit and Luminex technology (Linc Research, Millipore, Billerica, MA). Plasma norepinephrine and epinephrine were quantified using the ESA plasma catecholamine methodology and ESA Coulouchem LC/EC system (45–0161, Chelmsford, MA).

Serum zinc-alpha 2 glycoprotein (ZAG) was quantified by Western blotting. Antibody raised against a peptide mapping near the C-terminus of rat ZAG was purchased from Santa Cruz Biotechnologies (Santa Cruz, CA; ZAG-G-20:sc-11245). Serum was combined with Pro-Prep solution (Intron Biotech, Inc., Sungnam, Kyungkido), which included protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 0.7 μg/ml leupeptin, and 2 μg/ml pepstatin A, 0.5 μg/ml leupeptin, and 2 μg/ml aprotinin) and then centrifuged at 13,000 rpm for 10 min to remove the insoluble fraction. Total protein concentration for each sample was measured using the Micro BCA protein assay kit (Pierce, Rockford, IL). A 10 μg protein sample was boiled for 5 min in Laemmli loading buffer and then the protein separated by SDS-PAGE on a 10% acrylamide gel. Nitrocellulose membranes were used to electrobolt the proteins, which were then stained with Ponceau S to confirm equal protein loading and transfer. Membranes were washed in TBE with 0.1% vol/vol Tris-Buffered Saline Tween 20 (TBS-T), blocked in 5% wt/vol nonfat milk in TBS-T buffer for 1 h, and then incubated with goat anti-ZAG (1:1000, Santa
Cruz Biotechnology) antibodies at 4°C overnight. Membranes were then washed repeatedly in TBS-T and incubated for 1 h at room temperature with a horseradish peroxidase-conjugated donkey anti-goat IgG antibody (Santa Cruz Biotechnology). Immunoreactive bands were detected using the enhanced chemiluminescence reagent (GE Healthcare, Piscataway, NJ) and exposed to CL-X Posure film (Pierce, Rockford, IL). The quantification of band intensity was performed using a densitometric analysis program (Quantity One, Bio-Rad Laboratories, Hercules, CA).

Lipolysis Assay

For this assay, 3T3-L1 cells were grown to confluency in 96-well plates with 10% calf serum in DMEM culture media supplemented with L-glutamine, sodium pyruvate, nonessential amino acids, and penicillin/streptomycin added. The cells were continued 2 days beyond confluency and then stimulated to differentiate with 0.5 mM 1-methyl-3-isobutylxanthine (IBMX), 1 µM dexamethasone, and 86 nM insulin for 3 days. Then the cells were exposed to 86 nM insulin for 3 days in 10% fetal bovine serum in DMEM culture media. Three days later, the media was switched to 10% fetal bovine serum in DMEM in which the adipocytes were maintained. We used the cells within 7 days of reaching maturity. For all experiments, the media was carefully removed from the cells, the cells were washed with sterile PBS twice, and then the appropriate mixture was applied.

First, the following groups were tested for their lipolytic potential: Day 10 TB, Day 10 NTB, Day 15 TB, Day 15 NTB, Day 15 PF, Day 21 TB, Day 21 NTB, and Day 21 PF. Fifty µl of serum was mixed with 150 µl of DMEM culture media with penicillin/streptomycin added. Twenty-four hours later, the media was removed and the glycerol content immediately quantified using an enzymatic assay (Sigma, St. Louis, MO or ZenBio, Research Triangle Park, NC). The amount of glycerol released was determined from the difference in glycerol content before and after 24 h on the adipocytes.

The lipolytic potential of the lipid and delipidated portion of the serum was tested to determine if the factor was a lipid. Serum lipid was isolated using the method described by Bligh and Dryer (31). The serum was delipidated using fumed silica. Each fraction was mixed with DMEM media (40% vol/vol) and then placed on the 3T3 adipocytes. Twenty-four hours later, the media was removed and the glycerol released determined as previously described. Tumor serum, from which the samples were made, was also tested.

A lipolysis time course was determined. Serum from Day 21 TB and Day 21 PF animals was pooled and combined with 3T3 adipocyte maintenance media devoid of serum. This was placed on 3T3-L1 adipocytes and 2, 4, 6, 8, and 24 h later, a small sample of the media was removed and analyzed for glycerol content. From this, glycerol release was calculated.

Serum (2 ml) from Day 21 TB animals was dialyzed overnight against 50 mM tris buffer, pH 7.5, and then mixed with 0.5g DEAE cellulose (DE52, Whatman, Florham Park, NJ) equilibrated to 50 mM tris pH 7.5. The supernatant (labeled supernatant from DEAE), which contains unbound protein, was removed, and protein bound to the DEAE was eluted with an increasing NaCl solution (50 mM NaCl, 100 mM NaCl, and 150 mM NaCl). These 4 fractions (DEAE supernatant plus 3 salt fractions) were dialyzed against PBS overnight (4 changes) and concentrated to the original serum volume (2 ml) using glycercol-free, 5,000 kDa molecular weight, cutoff filters (Millipore, Billerica, MA). These samples were mixed with DMEM media (40%, vol/vol) and placed on 3T3 adipocytes. After 24 h, the media was removed and the glycerol content quantified enzymatically. The ZAG content of these samples was determined as described earlier (see Blood Collection and Analyses). Tumor serum, from which the samples were made, was also tested.

Tumor Cells

Tumor cells were harvested from an MCA-induced sarcoma at the time of implant. Briefly, the cells were released by treatment with collagenase and then sterile filtered and plated. The cells were maintained in RPMI 1640 media containing fetal bovine serum (10%), penicillin, and streptomycin. At 80% confluency, the media was replaced with fresh media. This media was collected 24 h later, combined with the 3T3 media (40:60 vol/vol), and placed on 3T3-L1 adipocytes. Twenty-four hours later, this media was removed, quickly cooled, and glycerol content quantified. Also, we tested the glycerol-releasing capacity of media not previous placed on the cultured MCA-induced sarcoma cells. Also, we tested the 3T3-L1 adipocyte maintenance media before and 24 h after it had been on the 3T3-L1 adipocytes.

Statistical Analysis

Longitudinal observations of body weight, net body weight (body weight net of tumor burden), food intake, and energy measures were modeled statistically as repeated measurements. These statistical models included an additional random effect parameter to account for covariance among observations on matched animals. The fixed effects portion of the models was composed of a factorial arrangement of treatment level and study day.

Analysis of body composition, serum lipolysis, blood constituents, and metabolites (Tables 1 and 2) preceded using mixed effects models with a factorial arrangement of treatment level and study day upon which the animals were sacrificed as fixed effects, and included a random effect to account for the effects of the initial matching of animals. Statistical significance for comparisons between treatment groups on a particular study day was derived from tests of differences between marginal means; in all cases, significance was declared relative to a 5% Type I error rate, unadjusted for multiple comparisons.

The serum lipid and lipid-free fractions were compared with tumor serum using 1-way analysis of variance. The same analysis was used to compare the 5 groups generated with DEAE separation.
TABLE 1
Blood concentration of circulating metabolites, lipolytic hormones, and selected adipokines 15 and 21 days after tumor implantation or sham operation

<table>
<thead>
<tr>
<th>Blood Constituent</th>
<th>Day 15</th>
<th>Day 21</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TB</td>
<td>PF</td>
</tr>
<tr>
<td>Serum Triglycerides (mg/dl)</td>
<td>38.9 ± 3.1</td>
<td>27.1 ± 2.7</td>
</tr>
<tr>
<td>Norepinephrine (ng/ml)</td>
<td>11 ± 3</td>
<td>15 ± 5</td>
</tr>
<tr>
<td>Epinephrine (ng/ml)</td>
<td>11 ± 2</td>
<td>19 ± 8</td>
</tr>
<tr>
<td>Corticosterone (ng/ml)</td>
<td>711 ± 40</td>
<td>870 ± 50</td>
</tr>
<tr>
<td>ACTH (pg/ml)</td>
<td>128 ± 13</td>
<td>206 ± 38</td>
</tr>
<tr>
<td>Adiponectin (ng/ml)</td>
<td>4.6 ± 0.2</td>
<td>6.2 ± 0.3</td>
</tr>
<tr>
<td>Leptin (ng/ml)</td>
<td>2.5 ± 0.3</td>
<td>2.7 ± 0.4</td>
</tr>
<tr>
<td>Insulin (ng/ml)</td>
<td>0.56 ± 0.04</td>
<td>0.71 ± 0.07</td>
</tr>
<tr>
<td>Glucagon (pg/ml)</td>
<td>131 ± 9</td>
<td>141 ± 13</td>
</tr>
<tr>
<td>Insulin to Glucagon ratio</td>
<td>4.4 ± 0.3</td>
<td>5.4 ± 0.6</td>
</tr>
<tr>
<td>ZAG (arbitrary units/µg protein)</td>
<td>72 ± 13</td>
<td>72 ± 12</td>
</tr>
</tbody>
</table>

Note. Abbreviations are as follows: TB, tumor bearing; PF, pair fed; NTB, nontumor bearing; ACTH, adrenocorticotropic hormone; ZAG, zinc alpha-2 glycoprotein. Values are means ± SE. Within each day, groups with different subscripts are significantly different, P < 0.01. n = 8/group.

RESULTS

Changes in Body Weight and Food Intake With Tumor Growth
Changes in body weight and food intake as the tumor grew are shown in Fig. 1. Throughout the entire 24-day period body weight measurements were collected, the total body weight of the TB animals did not differ significantly from the NTB animals. However, the host body weight of the TB animals, that is, the weight of the TB animal minus the weight of the tumor, was significantly less than the NTB and PF animals 15 days after tumor implantation (Day 15). This difference continued and became significantly greater with each subsequent day.

Food intake for the TB and NTB animals is shown in bottom graph of Fig. 1. Food intake did not differ significantly among the groups until Day 18 when the TB animals consumed significantly less food than the NTB. This difference was further accentuated as tumor size increased.

The growth of the tumor is shown in the middle panel of Fig. 1. The tumor is less than 2.5% of body weight on Day 15 and becomes significantly greater with each subsequent day.

TABLE 2
Blood concentration of cytokines 15 and 21 days after tumor implantation or sham operation

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>NTB</th>
<th>Day 15</th>
<th>Day 21</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TB</td>
<td>PF</td>
<td>TB</td>
</tr>
<tr>
<td>IL-1β (pg/ml)</td>
<td>18 ± 12 (5/12)</td>
<td>39 ± 33 (3/5)</td>
<td>12 ± 9 (3/5)</td>
</tr>
<tr>
<td>rGRO/KC (pg/ml)</td>
<td>76 ± 3 (12/12)</td>
<td>131 ± 66 (5/5)</td>
<td>114 ± 45 (5/5)</td>
</tr>
<tr>
<td>rIL-10 (pg/ml)</td>
<td>19.5 ± 16.9 (2/12)</td>
<td>1.6 ± 1.0 (3/5)</td>
<td>1.9 ± 1.6 (3/5)</td>
</tr>
<tr>
<td>rIL-6 (pg/ml)</td>
<td>18 ± 21 (2/12)</td>
<td>30 ± 27 (4/5)</td>
<td>30 ± 49 (2/5)</td>
</tr>
<tr>
<td>rTNFα (pg/ml)</td>
<td>18 ± 21 (2/12)</td>
<td>23 ± 11 (4/5)</td>
<td>11 ± 5 (5/5)</td>
</tr>
</tbody>
</table>

All data are presented in the tables and figures as mean ± SE.

Abbreviations are as follows: NTB NTB, nontumor bearing; TB, tumor bearing; PF, pair fed; IL, interleukin; TNF, tumor necrosis factor. Values are mean ± SE. IL-1β, rGRO/KC, rIL-10, rIL-6, and rTNFα were quantitated using Luminex technology. For IL-1β, rGRO/KC, rIL-10, rIL-6, and rTNFα, the first number in parenthesis is the number of samples that were quantifiable; the second number is the number of samples measured. To calculate the mean and SD, a non detectable value was assigned zero. The day 15 and 21 NTB values were averaged together.
FIG. 1. Changes in body weight and food intake with tumor growth. The top graph shows the changes in body weight among the 3 groups (TB, PF, and NTB) and the tumor bearing animal’s weight minus the weight of the tumor. The middle graph shows the rate of tumor growth. The bottom graph shows food intake for the TB and NTB groups. The PF are not included since their food intake was the same as the TB except delayed by 24 h. Each arrow indicates the day when body weight or food intake was first significantly different ($P < 0.05$). Each subsequent day to the right of the arrows was significantly different among the groups. We have repeated this experiment 3 times with similar results each time. Values are means ± SE ($n = 8$ per group).

Three days passed between the start of the decline in the host’s body weight and a drop in food intake. Clearly, the decrease in body weight occurs first and is not the result of a decrease in food intake. The inclusion of the PF group to control for food intake clearly demonstrates the portion of weight loss due to a reduction of food intake or due to a growing tumor (Fig. 1). The TB animals lost more weight than can be accounted for solely from a reduction in food intake alone.

Changes in Body Composition

Comparisons of the fat content of the TB and PF animals are shown in Fig. 2. The fat content of the TB animals compared to the NTB and PF animals was significantly less on Day 15 when the first difference in TB host body weight was observed (Fig. 2A). This difference remained at Day 21. No significant difference in protein content was observed on Day 15 or Day 21. By Day 28, the protein content of the TB animals was less (Day 15: NTB 57 ± 3 g, PF 49 ± 4 g, TB 50 ± 4 g; Day 21: NTB 46 ± 4 g, PF 44 ± 3 g, TB 41 ± 2 g; Day 28: NTB 48 ± 4 g, PF 44 ± 4 g, NTB 35 ± 6 g).

The lipid and protein content of the tumor did not change from Day 15 to Day 21 (not shown), so this does not account...
for the difference in fat content of carcasses observed in the TB animals.

There was a negative correlation between host lipid content (Fig. 2B) and tumor size; the larger the tumor, the less host lipid. This was not true for host protein content (data not shown; y = -0.09x ± 49.9, P < 0.03, R² = 0.09), which was lost much more slowly. The rate of lipid loss (-0.25 g carcass lipid/g tumor weight) was 2.5 times more rapid than the rate of protein loss (-0.09 g carcass protein/g tumor weight).

The loss of lipid by the TB animals was the result of the adipocytes shrinking in size (Fig. 2C). The total number of adipocytes (3,400 ± 1,200 cells/mg tissue) did not change between Days 15 and 21. Instead, the size of the adipocytes shifted dramatically by Day 21 from a size of 50 to 90 µm to <10 µm.

**Energy Expenditure**

Figure 3 reports the energy expenditure (kcal/h) during the dark and light cycle for the 3 groups. The energy expended daily did not differ significantly among the groups regardless of the time of day.

The respiratory exchange ratio (RER) is reported as a cumulative frequency for the TB animals on the lower graph in Fig. 3. As the tumor grew and fat mass was lost, more fat was used as a fuel. This is demonstrated by a shift in RER from 0.85 on Day 10 to <0.8 on Day 20.

**Blood Metabolites and Constituents**

We further verified the early changes in total body lipid content by measuring free fatty acid turnover on Day 17. Free fatty acid turnover was significantly higher in the TB animals (10.9 ± 0.9 mmol/day, n = 10, P < 0.05) compared to the NTB animals (9.3 ± 0.3 mmol/day, n = 10). This increased rate correlated with a significant elevation in serum triglycerides for the TB animals on Day 15 (Table 1). This trend continued, and by Day 21, serum triglyceride levels were more than fourfold higher than the PF and NTB groups.

A number of hormones and cytokines have been identified that promote lipolysis. We examined selected representatives to determine if any may be involved in promoting the body fat loss we observed. No significant differences in serum levels of norepinephrine, epinephrine, and corticosterone were found early (Day 15) when the first decline in body weight was observed (Table 1). ACTH was significantly lower in the TB animals on Day 15 and not elevated. Adiponectin was significantly lower on Day 15, but a decrease in leptin was not observed until Day 21.

Although glucagon levels did not differ among the groups, insulin levels were significantly lower in the TB on Day 15 and 21, making the glucagon to insulin ratio lower. However, this trend was not significant on Day 15 when the first change in body fat mass was observed and prior to cachexia. Since insulin levels decreased as cachexia progressed, we calculated the insulin to glucagon ratio. This ratio changed significantly when the animals were cachectic but not before.

ZAG has been shown to have lipolytic activity, so we determined the levels of this protein at different time points. We found no difference in the amount of ZAG present in the sera among the 3 groups on Day 15 and Day 21 (Table 1). In addition to ZAG, we measured the serum concentrations for 5 different...
cytokines (Table 2). There were no significant differences among the groups on any day measured.

**Serum-Induced Lipolysis**

Sera from Days 10, 15, and 21 for TB, NTB, and PF animals was added to 3T3 adipocytes to quantify the lipolytic potential as measured by the release of glycerol (Fig. 4). Increased glycerol release was observed on Days 15 and 21 for the TB animals compared to the PF and NTB. Two lines of evidence suggest this release was not due to a reduction in food intake. First, on Day 15, when food intake had not yet decreased, the TB serum stimulated significantly more glycerol release than sera from PF and NTB animals. Second, the amount of glycerol released on Day 21 was almost twofold greater for the TB group compared to the PF group. Our time course study indicates it takes between 8 and 24 h for the TB serum to stimulate glycerol release (Fig. 4). Also, we tested the lipolytic potential of media that had been on cultured MCA-induced sarcoma cells for 24 h. The results of this experiment are shown in Fig. 4. The media exposed to the MCA-induced sarcoma cells stimulated a greater release of glycerol than naïve media that had not been exposed to any cells and media that had been exposed to 3T3-L1 adipocytes for 24 h.

**Unknown Lipolytic Factor or Factors**

First, we determined if the factor or factors responsible for stimulating lipolysis was a lipid or was present in the lipid-free fraction of the serum. As shown in Fig. 5, delipidated serum stimulated a greater release of glycerol than the lipid fraction. The delipidated fraction had 71% of the activity of the tumor serum. Then we determined the stability properties of the lipolytic activity in the lipid-free serum fraction. This fraction was heated at 90°C for 10 min, insoluble material was removed by centrifugation, and the supernatant was tested in the lipolysis assay. The amount of glycerol released was sixfold less than the amount of glycerol released by the serum and the delipidated fraction and similar to that observed from the lipid portion of the serum.

Because ZAG has lipolytic activity (27), we measured the ZAG content of our active fractions to determine whether the observed lipolysis could be attributed to ZAG (Fig. 5). The fraction with the greatest lipolytic activity contained no measurable ZAG; thus, we conclude that the lipolytic activity observed in the serum of tumor-bearing animals was not due to the presence of ZAG.

**DISCUSSION**

Using the MCA-induced sarcoma model, we investigated changes before and after the development of cachexia as the tumor grew. We report that a loss of body fat occurs early, before loss of lean tissue mass, and prior to a reduction in food intake. Our interest is in identifying the promoters of this fat loss. In this model, there were 3 days between the reduction in fat mass and the decrease in food intake, suggesting something other than the

**FIG. 4.** A: Serum lipolytic potential. The amount of glycerol released from 3T3-L1 adipocytes was quantitated following 24-h incubation with media containing serum from TB, NTB, and PF animals. Animals were fasted overnight. Within each day, TB are the solid bar, PF are the open bar, and NTB are the cross hatch bar. * P < 0.05 TB vs. PF or NTB. Values are mean ± SE (n = 8/group). B: Time course. Glycerol release was quantitated 2, 4, 6, 8, and 24 h after pooled serum from day 21 TB and day 21 PF animals was placed on 3T3 adipocytes. The amount of glycerol released was not significantly different until the 24-h time point. C: Lipolytic potential of MCA-induced sarcoma media. Media (RPMI-1640) used to maintain cultured MCA-induced sarcoma cells for 24 h was tested against naïve media (DMEM media control and RPMI-1640 media control) and media used to maintain 3T3-L1 adipocytes for 24 h (DMEM). The media from the cultured MCA-induced sarcoma cells stimulated a significantly greater release of glycerol than any of the other 3 treatments.
reduction in food promotes the loss of body fat. We hypothesize that the promoters of cancer cachexia are present well before cachexia is observed clinically.

The reduction in body fat prior to loss of lean mass observed in our model agrees with the most recent human study published by Fouladun and associates (22). They found fat mass was lost first and more rapidly than lean mass. In our model, as tumor size increased, more lipid was lost from the host’s carcass than protein, adipocyte size decreased, and more lipid was present in the blood. Lean mass was lost more slowly.

During the last several decades, a number of theories have been proposed to explain cancer cachexia. These have included 1) increased hypermetabolism, 2) decreased food intake, or 3) circulating lipid mobilizing factors. We have examined each of these to determine if they played a role in the increased fat mass loss we observed in this model.

Hypermetabolism is often used to refer to a resting metabolic rate greater than predicted for healthy individuals. This has been studied using a variety of different methods and approaches, and some studies have reported hypermetabolism in some cachectic cancer patients (32,33). However, most studies have reported hypo or normal metabolism for cachectic cancer patients (34). In our model, energy expenditure was not different among the 3 groups prior to or after the development of cachexia through Day 21. We report energy expenditure as kcal/h. Changes in active cell mass can mask changes in energy expenditure since energy expenditure is determined by lean mass. We did not find a difference in lean body mass in the TB animals from Day 15 to 21 when cachexia developed. The loss in body weight was primarily fat mass. So we did not find daily energy expenditure changed or explained the early decrease in body fat mass.

Decreased food intake does promote weight loss and could contribute to cachexia. In our model, we had a 3-day period between the first changes in body weight and body composition and a decrease in food intake. Certainly a decrease in food intake promotes some of the weight loss reported from Day 18 to 21. The PF group controls for this and shows the amount of weight loss that is expected for the decreased amount of food the TB animals ate. So the early changes in body weight and fat mass can not be explained by a decrease in food intake alone.

Since changes in energy expenditure and a decrease in food intake did not adequately explain the mobilization of fat and lean tissue in animal tumor models, this led to the conclusion that a circulating lipid-mobilizing factor or factors was responsible for this effect. This is supported by a previously published parabiosis study (25) in the same tumor model we used and many in vivo and in vitro models including the 3T3 adipocyte model. Since the parabiosis study (25), there has been a long-standing interest in identifying factors that promote lipolysis in cachectic cancer patients, and a number have been identified including toxohormone-L (35), azaftig (36), and zinc-α2-glycoprotein (37).

Tisdale and colleagues (26,27) isolated a lipid mobilizing factor from the urine of cachectic cancer patients and identified it as ZAG. This is a ubiquitously expressed protein (38,39), and mRNA expression is upregulated in the adipose tissue of cachectic mice (40). ZAG upregulates G protein expression, which stimulates production of cAMP and the release of glycerol (41). Glucocorticoids stimulate lipolysis by increasing ZAG (40).

In the MCA model, increased lipolysis does not appear to be dependent on ZAG. First, serum levels of ZAG are not increased; and second, ZAG-depleted serum still promotes lipolysis. Recently, two studies have been published suggesting the role of ZAG is not simply that of a lipid mobilizing factor. First, an immunohistochemical analysis of murine xenografts did not find ZAG involved in cachexia (42). Second, Rolli and coworkers
published the phenotype for mice with both ZAG alleles inactivated. These mice had increased body weight, most likely the result of reduced lipolysis. This does suggest ZAG is involved in lipid mobilization; however, the reduced lipolysis did not occur via the adrenoreceptor pathway as previously reported (44), nor were differences between +/+ and –/– mice observed for a number of lipid metabolism molecules, including hormone sensitive lipase, lipoprotein lipase, or fatty acid synthase.

Other hormones and cytokines circulate and are known to promote lipolysis including catecholamines, cortisol, natriuretic peptide, and insulin. We did not measure natriuretic peptides because the lipolytic effect of these peptides appears confined to primates (45). We do report levels for catecholamines and cortisol and did not find these adequately account for the lipolysis we observed.

Insulin provides a block to lipolysis. Reducing circulating levels of insulin can promote lipolysis, and administration of insulin has been suggested as an anabolic treatment for cancer cachexia. Londos et al. (46) showed that decreasing concentrations of insulin increases lipolysis, and this effect occurs within minutes. We show the serum must be present for more than 8 h for lipolysis to occur, suggesting insulin is not the primary factor or only factor involved in our model. The long time for induction of the effect suggests that the mechanism of the factor(s) described here may require gene transcription.

A number of cytokines have been associated with cancer cachexia, including TNF-α, interleukin-1 (IL-1), interleukin 6 (IL-6), interferon-gamma (INF-γ), and ciliary neurotrophic factor (CNTF). Administration of these cytokines to animals alone or in combination leads to anorexia, weight loss, protein and fat breakdown, increased cortisol and glucagon, decreased insulin, and elevated energy expenditure. However, in weight losing cancer patients, blood levels of these cytokines are rarely elevated. We found the same in our model, suggesting these particular cytokines do not circulate and promote lipid mobilization. They may act locally within adipose tissue.

In summary, as is observed in human cancer patients, fat mass is lost first and more rapidly than lean tissue in the MCA-induced tumor model. The adipocytes shrink, and blood levels of triglyceride increase. We investigated the known lipolytic hormones and cytokines to determine if these are involved in promoting the loss of lipid from the adipocyte and the early development of cachexia. None of the circulating factors we investigated promoted this loss, including the most recently identified lipid mobilizing factor, ZAG. We conclude that a previously unidentified factor, most likely a protein, promotes this loss of lipid from the adipocyte. These studies will form the basis for further research to isolate and characterize this lipolytic protein.

REFERENCES


46. L. O. BYERLEY ET AL.