Activation of Thermogenesis in Brown Adipose Tissue and Dysregulated Lipid Metabolism Associated with Cancer Cachexia in Mice.

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ABBREVIATIONS

AP2: fatty acid binding protein 2
BAT: brown adipose tissue
BMI: body mass index
CCS: cancer cachexia syndrome
CD36: cluster of differentiation 36
C26: colon 26 adenocarcinoma
C/EBPα: CCAAT/enhancer-binding protein alpha
CPT1a: carnitine palmitoyltransferase 1a
DGAT2: diacylglycerol O-acyltransferase
ELOVL3: elongation of very long chain fatty acids
FAS: fatty acid synthase
18FDG: [18F]fluorodeoxyglucose
GYK: glycerol kinase
IL-6: interleukin-6
INFγ: interferon gamma
LPL: lipoprotein lipase
TNFα: tumour necrosis alpha
UCP1: uncoupling protein 1
PBE: peroxisomal bifunctional enzyme
PPAR: peroxisome proliferator-activated receptor
PGC1α: peroxisome proliferator-activated receptor gamma coactivator 1-alpha
PET: positron emission tomography
RER: respiratory exchange rate

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ABSTRACT

Cancer cachexia/anorexia is a complex syndrome that involves profound metabolic imbalances and is directly implicated as a cause of death in at least 20-30% of all cancers. Brown adipose tissue (BAT) plays a key role in thermogenesis and energy balance and potentially contributes to the physiological perturbations associated with cachexia. In this study, we investigated the impact of cachexia–inducing colorectal tumour on BAT in mice. We found that brown adipocytes were smaller and exhibited profound delipidation in cachectic tumour-bearing mice. Diurnal expression profiling of key regulators of lipid accumulation and fatty acid beta-oxidation and their corresponding target genes revealed dramatic molecular changes indicative of active BAT. Increased Ucp1, Pbe and Cpt1α expression at specific points coincided with higher BAT temperatures during the dark cycle, suggestive of a temporal stimulation of thermogenesis in cachexia. These changes persisted when cachectic mice were acclimatized to 28°C, confirming inappropriate stimulation of BAT despite thermoneutrality. Evidence of inflammatory signaling also was observed in the BAT as an energetically wasteful, maladaptive response to anorexia during the development of cachexia.
INTRODUCTION

Cancer Cachexia Syndrome (CCS) is a progressive metabolic syndrome clinically characterized by profound weight loss, fat depletion, skeletal muscle wasting and asthenia, that are not solely attributable to inadequate nutritional intake (1). The incidence of this debilitating condition varies with tumour type, and is more common in patients with upper gastrointestinal, colorectal and lung cancer while uncommon in breast cancer. While cachexia is implicated in ~30% of cancer deaths, it is also responsible for reduced quality of life and increased health care costs in many other cancer patients (2). There are currently no prognostic tests to indicate which cancer patients are at risk of developing cachexia, and once clinical signs of cachexia are apparent there are no effective treatments.

The molecular mechanisms that produce the profound clinical manifestations of cancer cachexia have not been elucidated. However, it is suspected that interactions between tumors and metabolic organs, in particular muscle & adipose tissue, trigger catabolic events that promote asthenia and depletion of lipid stores (3). In addition, it is unclear whether fat loss is due to cachectic mediators produced by tumors or inappropriate action of endogenous regulators in neuro/endocrine organs. Both experimental and clinical studies implicate cytokines as mediators of catabolism during cancer cachexia (4, 5) and markers of systemic inflammation have recently been incorporated into consensus clinical definitions of cachexia in multiple clinical settings including cancer (6).

Adipose tissue plays a vital role in metabolic balance by acting as an energy reserve organ, providing stored lipids to other tissues during periods of fasting or increased metabolic demand. However, regions of BAT are not only lipid depots but can significantly contribute to energy expenditure through diet- and cold-induced thermogenesis. Activation of BAT thermogenesis is thought to be mediated by thyroid hormone and β3-adrenergic signaling...
pathways, which stimulate β-oxidation of fatty acids and heat production via the electron transport chain-uncoupling protein UCP1. Until recently, the physiological function of BAT was only thought to be relevant in small mammals and infants. However, recent reports demonstrated activated BAT in adult humans acutely exposed to cold conditions and in up to 20% of cancer patients using ¹⁸F-FDG PET scans (7). In addition, BAT activity correlates with BMI, with decreased BAT activation potential in obese vs. lean males (8-12). While BAT has emerged as having a significant role in regulating energy balance and fat accumulation in rodents and humans, its involvement in hypermetabolic diseases such as cancer cachexia has not been extensively studied.

Energy homeostasis in metabolic organs is controlled by central and peripheral circadian clocks through tight regulation of expression and activity of enzymes involved in metabolic pathways (13). Apart from the core CLOCK-BMAL1-Reverbα mediated transcriptional regulation, nuclear receptors have also been found to exhibit distinct diurnal expression patterns in liver, muscle and BAT (14). Aberrant circadian rhythms result in hyperphagia, obesity and features of the metabolic syndrome. High fat diets can also disrupt circadian rhythms, and therefore further contribute to the metabolic syndrome (15, 16). Apart from the ability of melatonin – a key factor in controlling diurnal rhythms –to reduce circulating levels of TNF and IL6 in advanced cancer patients (17), little is known about whether circadian regulation of metabolism occurs in cancer cachexia.

In this study we demonstrate activation of thermogenesis in BAT of cachectic mice bearing C26 tumours. The diurnal expression pattern of transcription factors and target genes involved in lipid metabolism and heat production in BAT showed distinct temporal changes that coincide with increased BAT temperature. Therefore, the development of cancer cachexia involves disruption of diurnal regulation of lipid homeostasis and thermogenesis in
BAT. We also demonstrate evidence of cytokine signaling in BAT tissue implicating a role for tumour induced systemic inflammation in modification of BAT physiology.
MATERIALS AND METHODS

Cachectic Colon 26 cells were kindly provided by AMGEN (USA). The non-cachectic variant of Colon 26 was supplied by Tohuku University (Japan). Both cell lines were grown and maintained in RPMI medium (Invitrogen) containing 10% fetal bovine serum (Invitrogen) and 100 μg/ml Penicillin / Streptomycin (Invitrogen) in a 5% CO₂ environment.

Animal Studies

Pathogen-free, 8-10 week-old male and BALB/c*DBA/1 (F1 Hybrid) mice were purchased from ARC, Perth (Australia), and kept at an ambient temperature of 22°C under a 12 hour light cycle (6:00 am to 6:00 pm). Cachectic and non-cachectic colon 26 cells were inoculated subcutaneously at 1x10⁶ cells/100 μl into the right flank of mice. Control mice were similarly injected with 100 μl of RPMI medium containing antibiotics. Over a period of 14 days following inoculation, body weight, food intake and tumor dimensions were recorded daily. Tumor-bearing and free-fed control mice had ad libitum access to food. One group of control mice was pair-fed to the food intake of cachectic C26-bearing group. Pair feeding was achieved by giving each pair-fed control the previous days intake of its respective C26-bearing animal. Mice were euthanased by cervical dislocation before harvesting tumors and organs, which were snap frozen in liquid nitrogen. For the thermoneutrality experiments mice were acclimated for 5 weeks at 28°C prior to inoculation with either cachectic colon 26 carcinoma cells or RPMI medium. The experimental protocol was then followed as described above with the exception that the mice continued to be housed at 28°C for the duration of the experiment until harvest. All animal experimentation was carried out according to the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes under the Animal Research Regulation (2005) of NSW and under a protocol approved by the Animal Welfare Committee of Sydney South-West Area Health Service.
Plasma cytokine analysis

Plasma was collected from control, pair-fed and C26-bearing mice (cachectic and non-cachectic clones) via cardiac puncture and placed in EDTA or heparin coated sample tubes to prevent clotting. IL-6, TNFα and INFγ levels were measured using the RnD Biosystems Quantkine Mouse IL-6, TNFα and INFγ ELISA Kits respectively (Bioscientific Australia).

Indirect calorimetry studies.

Oxygen consumption rate ($V_{O2}$) and respiratory exchange rate (RER) of individual mice was determined using an eight-chamber indirect calorimeter (Oxymax series; Columbus Instruments, Columbus, OH) as previously described (18).

Biotelemetry

Mice were anesthetized using 5% isoflurane in oxygen in an induction chamber, then maintained on 2% isoflurane delivered by face mask. Temperature sensitive transmitters (E-mitters, Mini Mitter, Bend, OR) were implanted into the interscapular BAT of the mice who had previously had intracerebroventricular (ICV) cannulae implanted. Mice were shaved and a 1 cm incision was made just posterior to the scapulae to reveal the hindmost section of the white fat overlaying the interscapular area. A pocket was blunt dissected on the ventral side of the white fat to access the brown fat underneath. The E-mitter was then placed in the exposed pocket and the white fat sutured around the device. The wound was then sutured closed. After surgery, the animals received a single subcutaneous dose of 5 mg/kg meloxicam (Metacam, Boehringer Ingelheim) in a volume of 1 ml/kg and were maintained on a heated pad until full recovery. Mice were then allowed at least four days recovery before ICV injections and recording began. Temperature and activity data were collected via receiver plate below the home cage and recorded on a PC.
Adipose Tissue Histology and Microscopy

Brown adipose tissue samples were fixed in 10% formalin neutral buffered solution (Sigma-Aldrich), embedded in paraffin wax, and 5-μm sections cut and mounted on glass slides. After dehydration, the sections were stained with hematoxylin-eosin (H/E) for histological examination. Histology was viewed by light microscopy and examined by CAST grid software (Olympus Corp., Albertslund, Denmark). For electron microscopy brown adipose fat pads were fixed in 2% Glutaraldehyde in PBS for 2 hours. The fixative was then removed and tissue was rinsed with PBS. Fixed tissue was osmicated (1% OsO4/0.1 mol/L sodium cacodylate buffer), dehydrated in an ethanol gradient to 100% and embedded in Spurr's resin. Ultrathin sections (80nm thick), were obtained and viewed on a Phillips CM10 transmission electron microscope.

Quantitative Real-Time PCR analysis

Total RNA was isolated using Trizol (Invitrogen) according to the manufacturer’s protocol. First-strand cDNA was synthesized from 1 μg of total RNA using SuperScript III with random primers (Invitrogen) and oligo(dT)12-14 (Invitrogen). All primer sequences were BLASTed against the NCBI mouse genomic sequence database to ensure specificity for the corresponding gene. Primer sequences will be provided upon request. qPCR reactions contained 1.25 ng cDNA, 300 nM gene-specific primers and 12 μl SybrGreen (Invitrogen). All reactions were performed using the Corbett Rotor Gene 6000 (Corbett Life Science). Relative mRNA levels were calculated by the comparative threshold cycle method by using Tfec, Hmbs and 36b4 genes as the housekeeping controls (19). The results were then expressed as fold changes of Ct (cycle threshold) value relative to controls.
Western blotting

Whole-cell extracts were obtained using Cell Lysis Buffer according to manufacturer protocol, (Cell Signaling Technologies) and were quantified by BCA protein acid assay kit (Pierce). Mitochondrial extracts were obtained as described in (20). Proteins were resolved on 10% Tris-HCl SDS-PAGE gels (Bio-Rad), and blotted (iBlot, Invitrogen) as described by manufacturers. Cell lysates were analyzed with the following antibodies: anti-signal transducer and activator of transcription 3 (STAT3) and anti-phospho-STAT3-Ser727, anti-uncoupling protein 1. All antibodies were obtained from Cell Signaling Technology. Densitometric analysis was performed for western blots from three independent experiments using ImageJ software. Expression values of phosphorylated signals were normalized with corresponding total expression and indicated as fold change over the controls.

Statistical Analysis

Data are presented as the mean ± S.E.M. One-way ANOVA followed by Tukey’s multiple comparison tests were used to assess statistical significance between groups. Differences at $P < 0.05$ were considered to be statistically significant. ANCOVA was performed using SPSS for Mac OS X v16.0.1 (SPSS Inc, Chicago, IL, USA) with the final body mass as covariate and n=7 mice for each group. Other calculations were performed with Minitab statistical software.
RESULTS

Colon-26 adenocarcinoma affects body weight, food intake and brown adipose tissue mass while maintaining energy expenditure. A significant loss of body weight (Fig. 1A), accompanied by decreased food intake and BAT mass (Fig. 1B-C), occurred 14 days after C26 tumour inoculation. In contrast, pair-fed controls experienced only a moderate reduction in body weight and BAT mass indicating that anorexia alone could not completely account for the weight change in cachectic mice (Fig. 1A-B). To differentiate the impact of cachexia from the indirect effects of reduced food intake on energy expenditure, oxygen consumption and respiratory exchange ratio (RER) were measured in C26-tumour bearing mice, calorie-restricted and control mice. Diurnal oxygen consumption was identical between control and cachectic animals (Fig. S1A) while RER reduced to ~0.72 in C26 tumour-bearing animals, indicating lipids as the predominant fuel source (Fig. S1B). The effect of food-restriction on energy expenditure was examined over a shorter period of time, in an effort to minimize the technical difficulties in precise food delivery to pair-fed animals housed in metabolic cages. Non-tumour bearing mice were presented with the same amount of food ingested by C26 tumour-bearing mice corresponding to days 11, 12 and 13 post-tumor inoculation. A striking reduction in oxygen consumption in food-restricted animals during the entry to light cycle (Fig. S1C) coincided with milder reduction in RER compared to cachectic mice (Fig. S1D). When energy expenditure/heat production was calculated as kcals/mouse over the final 3 days of C26 tumor growth or food restriction, both groups were less than the corresponding control mice (Fig 1D and 1E). This is likely due to the progressive reductions in body weights in cachectic and calorie-restricted mice compared to ad lib fed control mice. However when the total energy expenditure over the final 12hr of the testing period is compared using ANCOVA with final body weight as covariate, there is a significant difference between cachectic C26 and pair-fed mice at the common body weight of 24.4g.
Cytokine signaling in BAT of cachectic C26 tumour-bearing mice. It is widely believed that pro-inflammatory cytokines are crucial for the development of cancer cachexia (6). We therefore examined the levels of IL-6, TNFα and INFγ in the plasma of C26-bearing mice. Consistent with previous studies, IL-6 concentrations were elevated in plasma from cachectic mice (Fig. 2A) while TNFα and INFγ were below the detection limit (data not shown). Increased expression of the IL-6 receptor and SOCS3 genes was apparent in BAT of C26 tumour-bearing mice (Fig. 2C-D). Further evidence for cytokine signaling via the JAK/STAT pathway was shown by substantially higher phosphorylation and hence activation of STAT3 within BAT of cachectic C26 mice compared to the minor change apparent in pair-fed mice (Fig. 2D). In order to differentiate the effect of tumour-derived cytokines on BAT from a non-cachectic cancer setting, we investigated the impact of a variant of the colon 26 tumour cell line that does not elicit cachexia. Despite the comparable growth of the non-cachectic C26 tumor, plasma IL6 levels were significantly lower while overall body, BAT weights and mRNA expression levels of IL6Ra and SOCS3 were unchanged (Fig 3.).

Perturbed diurnal expression pattern of lipid uptake and accumulation pathways in cachectic mice. To examine the impact of cancer-induced cachexia on lipid handling and de novo synthesis of fatty acids in brown adipocytes, we investigated the diurnal expression profile of metabolic genes and nuclear receptors at 6 timepoints in the light-dark cycle. Lipoprotein lipase (LPL), the major enzyme involved in hydrolysis of lipoproteins to release
fatty acids for uptake into cells, normally exhibits a daily rhythm in BAT reaching its maximum peak during the dark cycle at 10pm (Fig. 4A). Cachexia resulted in four-fold higher expression as well as loss of cycling of Lpl. There was no significant impact of cachexia on fatty acid transporter CD36 or carrier protein AP2. In control mice, Pparγ and associated target genes in lipogenic and adipogenic pathways peaked during the light cycle, while in the tumor-bearing animals Pparγ, C/ebpα, Fas, Dgat2 and Perilipin lost rhythmicity and were markedly decreased in BAT at several time points (Fig. 4B).

**Altered diurnal expression of lipid utilization and thermogenesis pathways in cachectic mice.** An important function of BAT is dissipation of energy in the form of heat through activation of fatty acid β-oxidation and thermogenic pathways. To understand the role of BAT in energy expenditure during cancer cachexia, we investigated the diurnal expression of transcription factors involved in regulating fatty acid catabolism and corresponding target genes (Fig. 5A). Pparδ normally has a diurnal oscillation peaking at 10am, while in cachectic mice this diurnal cycling was disrupted with higher expression at all time points. Pgc1, a key regulator of fatty acid metabolism, mitochondrial biogenesis & thermogenesis, maintained its diurnal peak at 10am but was elevated at most timepoints (Fig. 5A). PPARδ target genes peroxisomal bifunctional enzyme (PBE) and mitochondrial carnitine palmitoyl transferase 1α (Cpt1α) exhibited a similar diurnal peak at early dark cycle (6pm) with a 3-4 fold amplitude in healthy mice. BAT from cachectic mice showed higher abundance of both Pbe and Cpt1α mRNAs, as well as a significant 12-hour phase advance peaking at 6am. There was no change in expression of Pbe, Pgc1 and Pparδ in pair-fed mice (supplementary Figure S1).
Consistent with previous studies (17), key mediators of the thermogenic program in BAT exhibited oscillatory patterns at specific times of the diurnal cycle (Fig. 5B). β3 adrenergic
receptor (β3AR) transcripts peaked during the light cycle, however in cachectic animals this rhythm was attenuated and expression levels were reduced. Mediators of β3AR downstream signaling - adenylate cyclase 3 (Ac3) and deiodinase type 2 (Dio2) - behaved similarly at the mRNA level in healthy animals, peaking at 6pm. But in cachectic mice, the rhythmic expression was disrupted and was significantly higher at all time points. In control mice, the essential mediator of heat production in BAT – Ucp1 - displayed a diurnal pattern similar to β3AR. However in cachectic mice, the Ucp1 transcript lost its daily oscillation with maximal expression shifted towards the dark cycle. In contrast in pair-fed mice Ucp1 expression was reduced at 2pm (Fig. S1). These data provide compelling evidence that cachectic animals exhibited increased expression of genes involved in energy expenditure and non-shivering thermogenesis. Furthermore, the expression of genes such as Ucp1 involved in lipolysis and thermogenesis is unaltered in BAT of mice with the non-cachectic C26 tumor (Figure S2) that doesn’t produce IL-6 (Figure 3).

To determine whether these changes in gene expression coincided with heat production in cachectic animals we assessed BAT temperature using biotelemetry devices (Fig. 5C-D). Implanted telemetry devices recorded that C26 tumour-bearing mice exhibited elevated BAT temperatures, particularly during the dark cycle, between days 10-14 (Fig. 5C). BAT temperature was ~3-4°C higher in cachectic mice than in the pair-fed group. Pair fed animals had the lowest temperatures, which is in accord with the expected response to conserve energy during caloric restriction. In addition, physical activity appeared unchanged or even slightly reduced on days 11-13 in the cachectic mice indicating the source of increased temperature is unlikely to be heat from skeletal muscle activity (Fig. 5D). In contrast, pair-fed animals exhibited excessive physical activity despite reduced BAT temperature, likely due to foraging behavior. Therefore, activation of BAT in C26 tumour-bearing mice leads to inappropriate production of heat.
Activation of thermogenic program in BAT of cachectic mice persists at thermoneutral temperatures. To determine whether activation of BAT is due to an inability to maintain core body temperature when cachectic mice are housed under the mild cold stress of 22°C, we acclimatized the cachectic C26 mice to thermoneutral conditions - defined as a temperature (e.g., 28°C for mice) at which thermogenesis from mitochondrial electron transport chain uncoupling in BAT is not required. Similar to the previous experiments with mice housed at 22°C (Fig. 5), \( \beta_3 \)ar transcripts remained at lower levels in C26-bearing animals acclimated to thermoneutrality than in free- and pair-fed controls (Fig. 6). Ucp1, Ac3 and Dio2 exhibited significantly higher levels in cachectic mice, unlike food-restricted animals. In contrast to experiments performed at 22°C, Ucp1 expression at 28°C was significantly higher at both 2pm and 2am. Other genes known to be up-regulated during adaptation to cold conditions such as glycerol kinase (GYK) and elongation of very long chain fatty acids-like 3 (Elovl3) were also increased in cachectic mice at 28°C.

We assessed UCP1 protein abundance to determine whether the cumulative, temporal-specific increments in Ucp1 mRNA in BAT during the development of cachexia results in a net increase of UCP1 protein. Mitochondrial protein preparations of BAT from cachectic animals contained significantly more UCP1 protein compared to controls, while pair-fed mice had decreased mitochondrial UCP1 protein (Fig. 6B-C). Interestingly, the total mitochondrial protein yield per BAT in pair-fed mice was significantly reduced relative to control mice (Fig. 6D), presumably as part of the normal response to conserve energy by dampening BAT activity when calorie intake is chronically restricted. Therefore, when total UCP1 protein content is expressed per BAT organ (Fig. 6E), the increase exhibited by cachectic C26 tumour-bearing mice is even more striking when compared to pair-fed mice.
DISCUSSION

The importance of weight loss in cancer has been recognized for many years and has been generally attributed to muscle wasting and fat depletion (21). Earlier studies investigated limited molecular features of BAT in tumor-bearing or cytokine-administered animals (22-27). However, no systematic evaluation of lipid regulatory and metabolic genes in BAT, combined with energy expenditure and temperature assessment, has been performed in mice that exhibit sufficient features of cancer cachexia to satisfy the consensus clinical definition (6). Furthermore, diurnal regulation of thermogenesis in BAT has not been considered in cancer or other disease settings. We demonstrate, for the first time, thermogenic activation of BAT in cachectic animals that cannot be attributed to the effects of reduced food intake or inability to maintain core body temperature.

Since anorexia is associated with cachexia in cancer patients, we assessed changes in pair-fed animals presented with the same progressive food reduction as cachectic mice. The profound delipidation of BAT and increased expression of molecular regulators of lipid metabolism and target genes were not evident in pair-fed mice (Fig. 6 & S2). Indeed, UCP1 - the hallmark of thermogenic activation in BAT - was reduced at both mRNA and protein levels in response to food restriction while being increased in cachexia (Fig. 6). The observation that anorectic C26 cachectic mice maintain whole body energy expenditure at a higher level than corresponding sized calorie-restricted mice (Fig 1F & S1) while showing increased BAT and body surface temperatures provides further evidence of energetically wasteful processes in cachectic animals. This directly contrasts the effort to conserve energy in response to reduced calorie intake observed in pair-fed mice (Fig. 1 & 5). This apparent maladaptive response to caloric restriction in cachexia could have clinical relevance, as hypermetabolism is prominent in certain cancers such as lung, pancreatic, and leukemia (28, 29). The changes in the diurnal pattern of gene expression in BAT of cachectic mice are striking, especially the
increases in \textit{Pbe}, \textit{Cpt1}\textsubscript{\alpha} and \textit{Ucp1} that coincide with elevated BAT temperatures during the dark cycle when heat generation from increased physical activity would normally diminish the requirement for BAT-mediated thermogenesis. To our knowledge this is the first demonstration of altered diurnal expression of genes involved in lipid metabolism and thermogenesis linked to diurnal increases in BAT temperature due to cancer. Most of the genes examined in this study normally exhibit a diurnal rhythm, peaking either during the light cycle or at entry to the dark cycle (14). A recent study by Zvonic et al (30) found ~5,000 genes rhythmically expressed in BAT indicating synchronization of diurnal functions with physiological processes mediated primarily by circadian clock regulators and nuclear receptors (31). C26 tumour-bearing mice exhibited disrupted diurnal rhythmicity and phase shifting in BAT indicative of diminished \textit{de novo} synthesis and storage of lipids (\textit{Ppar}\gamma, \textit{C/ebp}\alpha, \textit{Fas}, \textit{Dgat2}, \textit{Perilipin}), increased uptake of lipids for $\beta$-oxidation rather than storage (\textit{Lpl}) and energy expenditure (\textit{Ppar}\delta). It will be interesting to explore the interplay of cytokine & neuro-endocrine signals responsible for the temporal coupling of increased BAT temperatures with up-regulated \textit{Ucp1}, \textit{Pbe} and \textit{Cpt1}\textsubscript{\alpha} expression alongside the uniform changes in thermogenic regulators \textit{Pgc1}\alpha, \textit{Ac3}, $\beta$3\textsubscript{ar} and \textit{Dio2}.

Our initial findings of activation of BAT in cachexia were derived from experiments with tumour-bearing mice housed at 22ºC, a temperature that elicits mild chronic cold stress. Therefore, it is possible that this response may be due to an inability of cachectic mice to maintain core body temperature, as observed in hypothermic MAC16 tumour-bearing mice that had increased UCP1 (32). To distinguish whether the observed changes in BAT are due to such a compensatory adaptation or a more active signal emanating directly from the tumour, we housed the cachectic C26 mice under thermoneutral conditions - defined as a temperature (eg 28-30ºC for mice) at which obligatory heat dissipation is sufficient to defend normothermic body temperature without thermoregulatory heat production. The changes in
cachectic mice persisted at thermoneutrality (Fig. 6) and were even greater than those apparent at 22°C relative to both control and pair-fed mice. Furthermore, expression of 2 genes associated with BAT-hypertrophy during cold conditions (33), Elovl3 and Gyk were also increased. The value of performing such investigations of complex metabolic syndromes involving energy imbalance at thermoneutrality is reinforced by recent studies with Ucp1 knockout mice that defined a role for UCP1 in diet-induced thermogenesis (34). This finding had eluded previous attempts at uncovering a phenotype for UCP1 when mice were acclimated to normal animal housing temperatures (35).

While the type of tumour, stage and duration of disease can influence resting energy expenditure, the systemic inflammatory status may also play an important role as high levels of cytokines and acute phase proteins have been observed in hypermetabolic patients with lung and pancreatic cancer (36, 37). Since cytokines are considered etiologic factors of cancer-induced energy wastage, as well as part of the consensus clinical definition of cachexia (5, 6), we investigated their potential involvement in the C26 model and observed plasma IL-6 levels within the range reported in cancer patients. IL-6 may enhance thermogenesis via direct action on BAT or by sympathetic nervous system (SNS) stimulation. For example, ventricular administration or hypothalamic expression of IL-6 increased energy expenditure, lipolysis & UCP1 expression via the SNS (38, 39). However, IL-6 induced fat-burning can also be exerted by an SNS-independent mechanism such as AMPK activation (40). Another pathway by which IL-6 could mediate direct effects on BAT is via MAP kinases. In response to SNS stimulation catecholamines signal through β-adrenergic receptors to enhance thermogenesis in BAT by activating p38 MAPK, thereby increasing mitochondrial biogenesis, expression of Pgc1α and Ucp1 and uncoupling (41, 42). The recent demonstration of a parallel pathway involving systemic cardiac derived natriuretic
peptides, cGMP and protein kinase B, that also operates via p38 (43) further highlights the importance of MAPKs in BAT thermogenic regulation. As IL-6 is a potent activator of MAPKs via Ras/raf, it may bypass the β-adrenergic and natriuretic protein receptors and their downstream effectors cAMP/PKA and cGMP/PKG to up-regulate UCP1. Increased UCP1 mRNA and protein abundance may augment net UCP1 uncoupling activity promoted by elevated fatty acid availability in brown adipocytes due to enhanced lipolysis.

While the effects of IL-6 through the SNS have not been investigated in the present study, our work supports the concept of cytokine-driven hypermetabolism and fat depletion in the cachectic animals. The exploration of the differences between cachectogenic and non-cachectogenic variants of the colon 26 tumour provides further intriguing evidence of cytokine involvement in BAT activation. This is evident in the failure of the non-cachectogenic, C26 variant to affect BAT (Fig. 3 & S3) coupled with a lack of corresponding circulating cytokines. Further studies into IL-6 or other cytokine signaling cascades operative in BAT of cachectic animals are required to define the links between tumour-derived cytokines and enhanced thermogenesis in cancer.

Clinical studies have found an association between cancer cachexia, systemic cytokines and fever (44). In particular, leukemia/lymphoma patients with B-symptoms (night sweats, weight loss and fever > 38°C) have elevated C-reactive protein together with reduced survival compared to patients without B-symptoms (45). These issues also occur in pediatric malignancies where there is greater concern due to the limited energy reserves and higher nutrient requirements in children (46). Despite the longstanding awareness of BAT in newborns and recent confirmation in adults through ¹⁸FDG PET imaging (8, 10, 11), there is no information about the association of active brown fat, plasma cytokines or CRP levels and weight loss in cancer patients. An early study found morphological evidence of brown adipose tissue in the peri-renal fat pad from 80% of cancer patients with cachexia (47).
view of the high prevalence of functionally active BAT in cancer patients, which approached 20% after serial PET scans (7), it will be interesting to correlate weight loss & inflammatory markers with activated BAT in sequential PET scans. Nevertheless, we cannot rule out the impact of other unidentified, yet tumor-derived factors or the involvement of SNS system on BAT activation.

In conclusion, based on these studies in a clinically relevant murine model of cachexia, reduced food intake is not the sole mechanism leading to weight loss during the development of cachexia. Rather, dysregulated diurnal expression of transcription factors that control lipid metabolism and thermogenesis in brown adipose tissue are likely to contribute to the fat depletion, elevated temperatures and hypermetabolic state of cancer cachexia.

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FIGURE LEGENDS

Figure 1. Metabolic parameters of mice bearing the Colon-26 tumour. (A) Body weight difference at day 14 (*P<0.05; ***P<0.001 vs initial body weight) (B) BAT adipose tissue weight changes at day 14 (**P<0.01; ***P<0.01 C26 vs control; ¥¥¥ P<0.001 C26 vs pair-fed); (C) Food intake at day 14 (**P<0.01 C26 vs control); (D) Energy expenditure in cachectic animals corresponding to days 11-13; (E) Energy expenditure in pair-fed mice corresponding to days 11-13; (F) Total energy expenditure during the final 12 hours of day 13 calculated by ANCOVA using final body mass as a covariate (¥¥ P<0.01 C26 vs pair-fed). (G) Haematoxylin/Eosin staining of BAT in (i) control free-fed, (ii) control pair-fed, (iii) C26 mice, Black bar represents 50 μm; (H) Electron microscopy images of BAT from (i) control free-fed, (ii) C26 mice. Black bar represents 2 μm, L lipid droplet, M mitochondrion; For A, B and C values are presented as a mean ± s.e.m. for 8-10 animals per group.

Figure 2. STAT3 signalling pathway is active in the BAT of cachectic mice but not in food-restricted mice; (A) Circulating IL-6 plasma levels from control, pair-fed and C26 tumour bearing mice (***P<0.001 C26 vs control). (B, C) mRNA analysis of Il6Ra and Socs3 isolated from BAT at 2am and 2pm from free-fed control (black circle), pair-fed control (green triangle) and C26 tumour-bearing mice (red square) (***P<0.01 C26 vs control). 2am values are duplicated in each graph to illustrate diurnal rhythmicity; (D) Protein levels of STAT3 and phosphorylated STAT3 (Ser727) in BAT of ad lib, pair-fed and C26 tumour–bearing mice assessed by immunoblotting. Protein extractions were performed in BAT harvested at 2 am. Values are mean ± s.e.m. presented as percentages relative to the controls for 4-6 animals per group (**P<0.01 C26 vs control).
Figure 3. Metabolic parameters of mice bearing the non-cachectic Colon-26 tumour and expression of genes involved in IL-6 signalling. (A) Body weight change between day 0 and day 14 (**P<0.001, cachectic C26 vs control), (B) BAT weight changes at day 14 ($P<0.05$ non-cachectic C26 vs control; ***P<0.001 cachectic C26 vs control); (C) Plasma analysis for IL6 cytokine from control, non-cachectic C26 tumour-bearing and cachectic C26 mice (**P<0.01 cachectic C26 vs control); (D-E) mRNA analysis of $IL6Ra$ and $Socs3$ isolated from BAT of free-fed control (black circle) and non-cachectic C26 tumour-bearing mice (orange square); 2am values are duplicated in each graph to illustrate diurnal rhythmicity. Values are mean ± s.e.m. presented as percentages relative to the controls for 4-5 animals per group.

Figure 4. Expression of genes involved in lipid uptake, trafficking and accumulation; mRNA analysis of $Lpl$, $Cd36$, $Ap2$, $Ppar\gamma$, $C/ebp\alpha$, $Fas$, $Dgat2$ and $Perilipin$ isolated from BAT of of free-fed control (black circle), and C26-bearing mice (red square). 6 am values are duplicated in each graph to illustrate a complete 24-h cycle; Values are mean ± s.e.m. presented as percentages relative to the controls for 4-5 animals per group. (* P<0.05; **P<0.01; ***P<0.001 C26 vs control).

Figure 5. Expression of genes involved in lipolysis and activation of thermogenesis in BAT; (A, B) mRNA analysis of $Ppar\delta$, $Pgcl$, $Pbe$, $Cpt1a$, $b3AR$, $Ac3$, $Dio2$, and $Ucp1$ isolated from BAT of free-fed control (black circle), and C26-bearing mice (red square); Values are mean ± s.e.m. presented as percentages relative to the controls for 4-5 animals per group (* P<0.05; **P<0.01; ***P<0.001 C26 vs control). 6 am values are duplicated in each graph to illustrate a complete 24-h cycle; (C, D) Changes in BAT temperatures and locomotor activity measured by implanted biotelemetry devices. Data represent the group
means of the temperature and physical activity for each animal at each time point for the final 7 days; C26-bearing mice (red), free-fed control (black), pair-fed control (green).

Figure 6. Expression of cold-adaptive genes and increased protein levels of UCP1 in brown adipose tissue under conditions of thermoneutrality; (A) mRNA analysis of B3Ar, Ac3, Dio2, Ucp1, Gyk and Elovl3 isolated from BAT of free-fed control (black circle), pair-fed control (green triangle) and C26-bearing mice (red square); 2am values are duplicated in each graph to illustrate diurnal rhythmicity. (B,C) Relative protein concentration of UCP1 in mitochondria isolated from cachectic, control and pair-fed animals; (D) Total mitochondrial protein content in C26-bearing, control and pair-fed mice; (E) Protein levels of UCP1 expressed per total mitochondrial protein content in BAT from cachectic, control and pair-fed animals; Values are mean ± s.e.m. presented as percentages relative to the controls for 4-5 animals per group (* P<0.05; **P<0.01; ***P<0.001 C26 vs control; ¥P<0.05; ¥¥P<0.01; ¥¥¥P<0.001 C26 vs pair-fed; §§ P< pair-fed vs control)
Figure 2

**A**

IL6 concentration (pg/ml)

**B**

Relative mRNA expression (%)

**C**

Relative mRNA expression (%)

**D**

Control | Pair-Fed | Colon-26

Coomasie stain

p-STAT3 (Ser727)

STAT3

Ratio P-STAT3 / STAT3

Figure 2
Figure 3

A. Body weight change (g) in control, C26 non-cachectic, and C26 cachectic groups.

B. BAT weight (mg) in control, C26 non-cachectic, and C26 cachectic groups.

C. IL6 concentration (pg/ml) in control, C26 non-cachectic, and C26 cachectic groups.

D. Relative mRNA expression of IL6Ra.

E. Relative mRNA expression of Socs3.
Figure 4
Figure 5
Figure 6
Activation of thermogenesis in brown adipose tissue and dysregulated lipid metabolism associated with cancer cachexia in mice

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