Heterogeneous time-dependent response of adipose tissue during the development of cancer cachexia

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Abstract

Cancer cachexia induces loss of fat mass that accounts for a large part of the dramatic weight loss observed both in humans and in animal models; however, the literature does not provide consistent information regarding the set point of weight loss and how the different visceral adipose tissue depots contribute to this symptom. To evaluate that, 8-week-old male Wistar rats were subcutaneously inoculated with 1 ml (2×10^7) of tumour cells (Walker 256). Samples of different visceral white adipose tissue (WAT) depots were collected at days 0, 4, 7 and 14 and stored at -80°C (seven to ten animals/each day per group). Mesenteric and retroperitoneal depot mass was decreased to the greatest extent on day 14 compared with day 0. Gene and protein expression of PPAR γ 2 (PPARG) fell significantly following tumour implantation in all three adipose tissue depots while C/EBP α

(CEBPA) and SREBP-1c (SREBF1) expression decreased over time only in epididymal and retroperitoneal depots. Decreased adipogenic gene expression and morphological disruption of visceral WAT are further supported by the dramatic reduction in mRNA and protein levels of perilipin. Classical markers of inflammation and macrophage infiltration (f4/80, CD68 and MIF-1 α) in WAT were significantly increased in the later stage of cachexia (although showing an incremental pattern along the course of cachexia) and presented a depot-specific regulation. These results indicate that impairment in the lipid-storing function of adipose tissue occurs at different times and that the mesenteric adipose tissue is more resistant to the 'fat-reducing effect' than the other visceral depots during cancer cachexia progression.

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Introduction

Cachexia is a complex metabolic syndrome that is a common end point shared by many chronic diseases, such as chronic heart failure, chronic obstructive pulmonary disease (COPD) and chronic renal failure (Tisdale 2010, Fearon 2011), but is most commonly observed in cancer patients. During the development of cancer cachexia, a well-characterised loss of both adipose tissue and skeletal muscle occurs (Dahlman *et al.* 2010, Arner 2011). It has been proposed that the former often precedes and is more rapid than the latter (Fouladiun *et al.* 2005). Several factors culminate in loss of adipose tissue, such as 1) increased lipid mobilisation due to enhanced adipocyte lipolysis (Agustsson *et al.* 2007, Ryden *et al.* 2008, Arner 2011), 2) reduced lipogenesis due to decreased lipoprotein lipase activity (Tisdale 2009) and 3) impairment of fat cell turnover, which results in a disruption in the formation and development of adipose tissue (Arner & Spalding 2010).

This knowledge should lead to a deeper understanding of the disease and development of appropriate interventions that might be more effective in preventing or delaying the onset of this syndrome.

Adiposity is controlled in part by alterations in the secretion of adipokines that control adipocyte metabolism as well as regulate diverse physiological processes in other tissues including the skeletal muscle, liver and brain (Fearon *et al.* 2006). In addition, adipose tissue mass is influenced by a finely regulated process of conversion of mesenchymal stem cells into mature adipocytes in response to a variety of extracellular effectors (Vernochet *et al.* 2009). These effectors coordinate the expression of an elaborate network of transcriptional factors that orchestrate the production of hundreds of proteins responsible for establishing the adipocyte lineage and subsequent differentiation (adipogenesis) into mature fat cells (Farmer 2005). At the centre of this process are the two principal adipogenic factors, PPAR γ (PPARG) and

C/EBP α (CEBPA), which captain the entire terminal differentiation process. PPAR γ is considered the master regulator of adipogenesis; precursor cells lacking this transcriptional factor are unable to develop into fully functional adipocytes capable of lipid metabolism and secretion of adipokines (Rosen *et al.* 2009). Interestingly, progenitor cells that do not express C/EBP α are still capable of differentiating into adipocytes; however, the resulting C/EBP α -deficient adipocytes are insulin resistant (Rosen *et al.* 2009). SREBP-1c (SREBF1) is an additional factor participating in adipocyte maturation by regulating the expression of genes encoding target enzymes involved in the biosynthesis of fatty acids.

Involuntary weight loss, such as that present in cancer cachexia, causes a decrease in the size of adipocytes without changes in their number, resulting in adipose tissue atrophy (Bing & Trayhurn 2009). Bing *et al.* (2006) showed that the epididymal adipose tissue (EAT) of cachectic mice contained shrunken adipocytes with dramatically reduced cell size that may have resulted from the down-regulation of key adipogenic and lipogenic factors. However, the authors have not provided any information regarding the onset of the syndrome or whether the dysfunction occurs in other adipose tissue depots.

White adipose tissue (WAT) is a heterogeneous tissue displaying marked depot-related specialisation in relation to many parameters, including cellularity, growth, metabolism, production of and response to cytokines as well as hormones, innervation and fatty acid composition (reviewed by Pond (1999, 2002)). Previous studies from our group using an animal model of cachexia have shown that different depots are heterogeneous and in a time-dependent fashion affected by the syndrome (Bertavello & Seelaender 2001, Machado *et al.* 2004). Nevertheless, whether there is a corresponding time-dependent and site-specific change in the way fat loss occurs is still controversial.

To gain greater insight into adipose tissue wasting during the development of cancer cachexia and into the associated morphological and molecular mechanisms, we have analysed different WAT depots at three time points following tumour injection. We herein present results regarding 1) the time course of adipose tissue atrophy during cachexia progression, 2) circulating adipokine profile, 3) changes in the levels of mRNA and in protein expression of key adipogenic factors and of factors controlling specific functions of the mature adipocyte and 4) the presence of inflammatory cells and the association between infiltration and the alterations of the other parameters. The analysis shows profound early alterations in the expression of major adipogenic and lipogenic transcriptional factors followed by morphological modifications of the adipocytes, which become more evident at the late stage of the disease. In addition, the results suggest that the mesenteric adipose tissue (MEAT) is more resistant to the 'fat-reducing effect' elicited by the tumour than the other visceral depots studied during the cancer cachexia progression.

Materials and Methods

Animals

Male adult Wistar rats (160–250 g), obtained from the Institute of Biomedical Sciences, University of São Paulo, were maintained in metabolic cages, in a 12 h light:12 h darkness cycle (lights on at 0700 h), under controlled temperature conditions (23 ± 1 °C), receiving water and food (NuvilabCR1-Nuvital[®], Curitiba, Paraná, Brasil) *ad libitum*. The Ethical Committee for Animal Research from the Institute of Biomedical Sciences, University of São Paulo approved all the adopted procedures, which were carried out in accordance with the ethical principles stated by the Brazilian College of Animal Experimentation.

Weight and food intake were assessed daily, always in the afternoon. Walker 256 tumour cells (2×10^7 cells) were injected s.c. into the right flank of the animals (Seelaender *et al.* 1998). Control rats received saline injections on the same day of tumour inoculation. Cachectic syndrome is observed in rats bearing Walker 256 tumour cells after 10–15 days (Bennani-Baiti & Walsh 2011). To evaluate the development of cancer cachexia, the experiments were carried out in the time course study, when the rats were killed on days 4, 7, 10 or 14 post-injection (seven to ten animals on each time point) by decapitation after 12 h fasting.

Blood samples and adipose tissue collection

Trunk blood was collected after decapitation into 15 ml conical bottom Falcon tubes containing EDTA (1.8 mg/ml of blood), centrifuged at 500 *g* and 4 °C for 10 min, and stored at -80 °C. EAT, retroperitoneal adipose tissue (RPAT) and MEAT (after careful removal of adjacent lymph nodes) were removed, weighed, snap frozen in liquid nitrogen and stored at -80 °C.

Plasma and tissue analysis

General plasma markers and adipokine concentration The concentration of triacylglycerol, glucose and total protein was measured by commercial kits. Adiponectin was measured by ELISA (EZRADP-62K, Millipore, Inc., Billerica, MA, USA) and leptin by RIA with the species-specific kit RL-83K (Linco Research, Inc., Saint Charles, Missouri, USA). The sensitivity of adiponectin assay was 0.15 ng/ml, and the inter-assay coefficient of variation (CV) ranged from 4.3 to 8.4%. The sensitivity of leptin assay was 1 ng/ml, and the inter-assay CV ranged from 3.0 to 5.7%.

Light microscopy After fragmentation and fixation (4% paraformaldehyde, pH 7.2) for 16 h at 4 °C, adipose tissue samples were dehydrated in absolute ethanol, cleared in xylene and then embedded in paraffin. The paraffin was cut into 5 mm sections that were stained with Harris

haematoxylin, counterstained with eosin and then analysed under light microscopy (Leica, DM 750). For the analysis of the morphometrical aspects, the area, approximate diameter, perimeter and shape factor were measured in 100 adipocytes (five slides per tissue from one rat, five animals per group). A total of 1500 cells per group were studied. Analysis of the results was performed using the Sigma ScanPro4 program.

Real-time PCR Total RNA was isolated from the EAT, RPAT and MEAT samples using TRIzol (Invitrogen) following the manufacturer's recommendations. The concentrations of RNA were determined by measuring the absorbance at 260 nm. The purity of the RNA was determined by calculating the absorbance ratio at 260 and 280 nm, and by ethidium bromide staining. RT-PCR was performed on the total RNA (4 µg), which was used for first-strand synthesis of cDNA using a commercially available kit (Ambion, Austin, TX, USA). The reaction mixture was stored at -80°C until the PCR step. Primer sets for rat perilipin (NM 013094.1: sense, 5'-GCC TAT GAG AAG GGT GTA CAG G-3' and antisense, 5'-CTT CAG TTC AGA GGC GAT CTT T-3'), PPAR γ 2 (NM 001145366.1: sense, 5'-CTG CCT ATG AGC ACT TCA CAA-3' and antisense, 5'-CAT CAC AGA GAG GTC CAC AGA G-3'), C/EBP α (NM 012524.2: sense, 5'-GAA GTC GGT GGA TAA GAA CAG C-3' and antisense, 5'-GGT CAT TGT CAC TGG TCA ACT C-3'), SREBP-1c (XM 213329.5: sense, 5'-AGG GAG TTC TCA GAT GCT CTT G-3' and antisense, 5'-CAT GCT GGA ACT GAC AGA GAA G-3'), leptin (NM 013076.3: sense, 5'-AGC TGT GCC TAT CCA CAA AGT CCA-3' and antisense, 5'-AAT GAA GTC CAA ACC GGT GAC CCT-3'), adiponectin (NM 144744.3: sense, 5'-ATC CTG CCC AGT CAT GAA GGG ATT-3' and antisense, 5'-TGC CAT CCA ACC TGC ACA AGT TTC-3'), resistin (NM 144741.1: sense, 5'-ACTA GCT GCT CCT GTG GCT CT-3' and antisense, 5'-CAG TCT ATG CTT CCG CAC TG-3'), haptoglobin (NM 012582.2: sense, 5'-CCT GCC TTC CAA AGA CTA CG-3' and antisense, 5'-CTC ACA CTT CTC CTG GTC AGC-3'), CD68 (NM 001031638.1: sense, 5'-ACA AAC AGT CCA GGC TTC TCC ACT-3' and antisense, 5'-TGG CTG GGA ACC ATT AGT CCA TGT-3'), F4/80 (NM 001007557.1: sense, 5'-CTC TTC CTG ATG GTG AGA AAC C-3' and antisense, 5'-CCC ATG GAT GTA CAG TAG CAG A-3') and macrophage migration inhibitory factor (MIF; NM 031051.1: sense, 5'-TCC CGG ACC ACG TCA TGA CTT T-3' and antisense, 5'-CGT TGG CTG CGT TCA TGT CGT AAT-3') were designed using Primer Express Software v2.0 (Applied Biosystems). The results for mRNA concentrations are expressed as a ratio over 18S rRNA, which was amplified as a housekeeping gene using the following primers: 18S rRNA (M11188.1: sense, 5'-TCA GCT TTG CAA CCA TAC TCC-3' and antisense, 5'-GAC CAT AAA CGA TGC CGA CT-3'). For each sample, PCR was performed in duplicate in a 25 µl reaction volume of 5–20 ng of cDNA, 12.5 µl Syber Green Master Mix

(Applied Biosystems), and 200–600 nM of each primer. PCR analyses were carried out using the following cycle parameters: 50°C for 2 min, 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Fluorescence was quantified and analyses of amplification plots were performed with the ABI Prism 7700 Sequence Detector System (Applied Biosystems). All samples were normalised to the 18S values and the results were expressed as fold changes of Ct value relative to controls using the $2^{-\Delta\Delta\text{Ct}}$ formula. 18S was used as a reference gene as we have experimentally determined that there were no statistically significant differences of its Ct values between control and different times from tumour-bearing groups (both $P < 0.05$, three experiments).

Western blotting Frozen tissue (0.1–0.3 g) was homogenised (1:5) in RIPA buffer (0.625% Nonidet P-40, 0.625% sodium deoxycholate, 6.25 mM sodium phosphate and 1 mM ethylenediaminetetraacetic acid at pH 7.4) containing 10 µg/ml of a protease inhibitor cocktail (Sigma–Aldrich). Homogenates were centrifuged at 12 000 g for 10 min at 4°C , the supernatants (fatty layer) were discarded and the infranatant was saved. Protein concentration was determined with a BCA protein quantification kit (Pierce, Rockford, IL, USA), with BSA as a reference. Samples containing 50 µg protein were separated by electrophoresis on a 10% SDS–PAGE. Proteins were then transferred to PVDF membranes at 100 V for 1 h in a transfer buffer containing 20 mM Tris, 150 mM glycine and 10% methanol. PVDF membranes were then blocked in TBS containing 0.1% Tween 20 and 5% milk for 1 h. After three washes with TBS–0.1% Tween, the PVDF membranes were incubated with primary antibodies against C/EBP α (polyclonal Abcam 74404) at a 1:5000 dilution, PPAR γ (polyclonal Abcam 19481) and perilipin (polyclonal Abcam 3526) at a 1:2000 dilution, and adiponectin (polyclonal Abcam 62551) at a 1:2000 dilution and hormone-sensitive lipase (HSL; polyclonal Abcam 45422), all over night. After three washes with TBS–0.1% Tween, the PVDF membranes were incubated with secondary antibodies goat anti-rabbit IgG conjugated to HRP (Santa Cruz Biotechnology, Inc.) at 1:5000 for 1 h at room temperature and were detected by ECL (Amersham, Little Chalfont, UK). For the control of protein equal loading, blots were stripped and then incubated with β -tubulin (polyclonal thermo RB-9249-P0). Quantification of antigen–antibody complexes was performed using Image J Analysis Software (<http://rsb.info.nih.gov/ij/>). Optical density units are expressed in pixel for fold target protein/control protein.

Statistical analysis Data are expressed as mean values $\pm$ S.E.M. Differences between time of tumour cell inoculation (cachexia development, days 0, 4, 7 and 14) were analysed by one-way ANOVA followed by Bonferroni's *post-hoc* comparisons tests using GRAPHPAD PRISM software for Macintosh, version 5.0 (GraphPad, San Diego, CA, USA). Differences between qualitative variables on days 7 and 14

against control (matched by age) were determined by the unpaired form of the Student's *t*-test, using the same software, and *P*<0.05 was considered significant for all statistical tests.

Results

Adipose tissue is not only considered the most important energetic reservoir of the body but is also recognised as an active endocrine tissue. Its functionality is affected by a set of physiological and non-physiological events such as cachexia, which is a syndrome frequently observed in cancer patients. The paraneoplastic disease causes lipodystrophy as part of the overall wasting of affected individuals. To investigate the effects of cachexia on adipose tissue with regard to disease course and anatomical-specific aspects, Walker 256 tumour cells were injected s.c. Tumour-bearing rats presented considerably reduced body weight compared with non-tumour-bearing controls (8.4%, *P*<0.05) on day 14, with no changes on day 7 (Table 1). Tumour burden on day 7 post-inoculation represents 8% and on day 14, 29% of total body mass. Fat mass weight (absolute and relative to body weight) was also markedly reduced in both MEAT and RPAT of tumour-bearing rats on day 14 when compared with non-tumour-bearing controls (absolute: 39% (MEAT) and 32% (RPAT), and relative: 31 (MEAT) and 26% (RPAT), both depots *P*<0.001; Table 1). There was no change in absolute and relative weight of EAT. Decreased chow consumption was evident from day 10 after inoculation in the tumour-bearing rats (data not shown).

Animals bearing the tumour showed statistical differences in absolute and relative weights of the different fat pads during the development of cancer cachexia, suggesting the possibility that modification of cell morphology contributes to these changes. In order to address this hypothesis, we determined cell perimeter and cross-sectional area among the three depots studied (Fig. 1). Light microscopy examination showed that

morphometric alterations of adipose tissue in tumour-bearing rats during the progression of cancer cachexia are depot specific (Fig. 1Aa, b, c, d, e, f, g, h and i). In MEAT, adipocyte size, determined as cell perimeter (Fig. 1B) and sectional area (Fig. 1C), showed an increase of 52% (*P*<0.05) and 1.3-fold (*P*<0.001) respectively on day 14. Conversely, the cell perimeter of RPAT and EAT adipocytes was reduced (54 and 27% respectively, *P*<0.05) as well as the sectional area (85 and 47% respectively, *P*<0.05) on day 14 compared with day 0. On the other hand, MEAT showed an unexpectedly opposite effect concerning morphology, i.e. increased perimeter and section area. These data illustrate a regional and heterogeneous fat distribution in tumour-bearing rats.

Considering that the diameter of cells measured in MEAT was slightly affected by the presence of the tumour and continued to enlarge during cachexia progression, we put forward the question whether protein and mRNA expression of perilipin, one of the major lipid-coating proteins, would also be increased (Fig. 2A). In this aspect, we also evaluated protein expression of HSL (LIPE), a key enzyme in the regulation of WAT lipolysis (Fig. 3). In MEAT, the reduction in perilipin mRNA levels was only evident on day 14 (76%, *P*<0.01). By contrast, perilipin mRNA levels in RPAT and EAT dramatically decreased by 92 (*P*<0.001) and 55% (*P*<0.01) on day 4; 97 and 77% on day 7; and 98 and 57% on day 14 respectively (*P*<0.001 for both days and tissues) compared with control (day 0). Perilipin protein levels were reduced only on day 14 in MEAT (91%, *P*<0.01) and RPAT (77%, *P*<0.01) compared with control (day 0, Fig. 2B). There was no change in perilipin protein expression in EAT. HSL protein expression was just affected by cachexia on day 14, showing increase of 64% in relation to day 0. There was no alteration on protein expression on RPAT and EAT considering the different time period evaluated.

To gain additional insight into the reasons why the tumour induced a regional response on fat cell diameter and number, we examined the expression of the major transcription factors

Table 1 General characteristics of animal groups during the progression of cancer cachexia. Values are mean ± S.E.M. for seven animals per group

	Tumour cell inoculation (days)			
	Day 7		Day 14	
	Control	TB	Control	TB
<i>n</i>	7	12	7	14
Body weight (g)	269.1 ± 19.4	265.9 ± 17.4	312.7 ± 11.6	286.4 ± 12.2*
Tumour weight (g)		3.0 ± 1.4		10.1 ± 6.4
MEAT (g)	1.3 ± 0.3	1.3 ± 0.3	2.5 ± 0.5	1.5 ± 0.2*
MEAT (% BW)	0.5 ± 0.1	0.5 ± 0.1	0.8 ± 0.2	0.5 ± 0.1*
RPAT (mg)	1.5 ± 0.4	1.4 ± 0.4	3.5 ± 0.5	2.1 ± 0.5*
RPAT (% BW)	0.5 ± 0.1	0.5 ± 0.2	1.1 ± 0.2	0.7 ± 0.2*
EAT (mg)	2.1 ± 0.4	2.0 ± 0.4	3.5 ± 0.4	3.0 ± 0.3
EAT (% BW)	0.8 ± 0.1	0.8 ± 0.2	1.1 ± 0.1	1.1 ± 0.1

TB, tumour-bearing; MEAT, mesenteric adipose tissue; RPAT, retroperitoneal adipose tissue; EAT, epididymal adipose tissue; BW, body weight. **P*<0.05 vs control.

Figure 1 Morphological characteristics of different adipose tissue depots during cancer cachexia progression. (A) Haematoxylin and eosin-stained sections of MEAT tissue from tumour-bearing rats at control (a), day 7 (d) and day 14 (g); RPAT at control (b), day 7 (e) and day 14 (h) and EAT at control (c), day 7 (f) and day 14 (i). Morphometrical analysis of sectional area (B) and (C) perimeter of adipocytes from different depots in different time period (0, 7 and 14 days after tumour cells inoculation). MEAT, mesenteric adipose tissue; RPAT, retroperitoneal adipose tissue; EAT, epididymal adipose tissue. Values are mean $\pm$ s.e.m. for seven animals per group. * $P < 0.05$ vs control (day 0). Full colour version of this figure available via <http://dx.doi.org/10.1530/EJE-12-0307>.

involved in fat cell formation, PPAR γ 2, C/EBP α and SREBP-1c (Fig. 2A). Protein and mRNA expression of PPAR γ 2 fell throughout the 14 days of the experimental protocol in the three depots. Regarding PPAR γ 2 mRNA levels in MEAT and RPAT, there were decreases of 75% ($P < 0.001$) and 57% ($P < 0.01$) respectively on day 4; in MEAT, RPAT and EAT, they dropped 38, 51 and 73% ($P < 0.05$) respectively on day 7 and 86, 72.6 and 82% ($P < 0.01$) respectively on day 14 compared with day 0 (Fig. 2A). PPAR γ 2 protein level was reduced in all evaluated depots at day 14 (MEAT, 56.7%; RPAT, 76% and EAT, 75%, $P < 0.01$; Fig. 2B). PPAR γ 2 protein level was reduced in all evaluated depots at day 7 (MEAT, 69%; RPAT, 83% and EAT, 74%, $P < 0.01$) and day 14 (MEAT, 57%; RPAT, 76% and EAT, 75%, $P < 0.01$) (Fig. 2B).

C/EBP α mRNA expression, another key regulator of fat cell formation, also declined over time but only in EAT and RPAT depots while in MEAT the expression remained stable. RPAT and EAT showed reductions of 84% ($P < 0.001$) and 62% ($P < 0.001$) on day 4 and 90 and 78% on day 7 respectively ($P < 0.001$ for both) (Fig. 2A). We also determined protein expression of the C/EBP α isoforms of 42 and 30 kDa in total extracts of the three depots (Fig. 2B). p42 C/EBP α expression levels decreased in RPAT at day 7 (51%, $P < 0.05$) and day 14 (48%, $P < 0.05$), while expression

of p30 C/EBP α was not detected at any of the times. In EAT, though, the pattern of expression was opposite: p30 C/EBP α was present on day 0 and down-regulated on day 7 (66%, $P < 0.05$) and day 14 (62%, $P < 0.05$), but p42 C/EBP α was detectable only on day 7. Moreover, the expression of p42 C/EBP α in MEAT increased progressively during the course of cachexia (day 7, 36%, $P < 0.05$ and day 14, 1.5-fold, $P < 0.01$), with no significantly consistent changes in p30 C/EBP α , suggesting that p42 C/EBP α is probably mediating the stability of fat cells in this depot. Finally, the mRNA expression of SREBP-1c, a transcription factor involved in aspects of adipocyte maturation, increased twofold ($P < 0.001$) in MEAT on day 14 compared with day 0, with no significant changes during the earlier time periods. In RPAT and EAT, there was a reduction of 86.9% ($P < 0.001$) and 80% ($P < 0.001$) on day 4; 46 and 73% on day 7 and 85 and 33% on day 14 respectively ($P < 0.001$ for both days and tissue) in relation to day 0.

Our next aim was to investigate whether the changes in PPAR γ and C/EBP α expression correlated with the alterations in the synthesis and secretion of adipokines by adipocytes into the circulation. Adipokines as well as metabolic parameters were evaluated during cancer cachexia progression (Table 2). Plasma glucose and total protein levels were reduced by 18% ($P < 0.01$) and

Figure 2 Expression levels of genes involved in relation to lipid-coating proteins and adipogenesis (A) in different adipose tissue depots during cancer cachexia development. Real-time PCR analysis of RNA isolated from MEAT, RPAT and EAT of control (day 0), 7 and 14 days after tumour cell inoculation. mRNA levels of target genes were normalised to 18S. Values are mean $\pm$ s.e.m. for seven animals per group. * $P < 0.05$ vs control (day 0). (B) Western blotting analysis of protein extracts from different adipose tissue depots. Blots were rearranged and most representative images were selected for perilipin, PPAR γ , C/EBP α and for SREBP-1c and β -tubulin proteins (normalised protein). MEAT, mesenteric adipose tissue; RPAT, retroperitoneal adipose tissue; EAT, epididymal adipose tissue. Values are mean $\pm$ s.e.m. for five animals per group. * $P < 0.05$ vs control (day 0).

19% ($P < 0.01$) respectively on day 14 compared with day 0. Total plasma triglyceride increased, 22% ($P < 0.05$) on day 7 and 30% ($P < 0.01$) on day 14, when compared with the control (day 0). Plasmatic values for adiponectin, an adipokine with effects on insulin sensitivity, were elevated by 54% ($P < 0.01$) on day 4, yet returned to basal values on day 7, finally decreasing by 26% ($P < 0.05$) on day 14. Resistin plasma levels rose about 1.8-fold ($P < 0.01$) on day 7, returning to basal values on day 14. Finally, we determined leptin plasma levels, an adipokine displaying a plethora of regulating effects on the control of energy intake and expenditure, among many others. Our results show a reduction of leptin levels on day 14. In addition, we evaluated the expression of adipokine genes including adiponectin, leptin, resistin and haptoglobin, as shown in Fig. 4. Adiponectin mRNA levels in MEAT showed an increase of four-, five- and two-fold ($P < 0.001$) on days 4, 7 and 14 respectively. RPAT and EAT levels were reduced by 57% ($P < 0.01$) and 60% ($P < 0.01$) on day 4; 51 and 84% on day 7

and 72 and 84% on day 14 respectively ($P < 0.001$ for both days and tissues) compared with day 0. Regarding adiponectin, we also determined its protein expression (Fig. 3). Adiponectin expression levels increased in MEAT at day 7 (1.1-fold, $P < 0.05$) and day 14 (70%, $P < 0.05$). In RPAT, there was a decrease at day 14 (54%, $P < 0.05$), while expression in EAT samples were not detected at any of the times. Resistin did not differ in any of the tissues in the study period, when compared with day 0 (not shown). Leptin did not change in MEAT in any time point. In RPAT, leptin expression showed a reduction of 67 and 55% ($P < 0.05$) on days 7 and 14 respectively compared with day 0. In EAT, leptin mRNA levels were lower on day 4 (60%, $P < 0.01$), day 7 (80%, $P < 0.001$) and day 14 (83%, $P < 0.001$) when compared with control. Haptoglobin showed an increase by three- and four-fold ($P < 0.001$) on day 7 in MEAT and RPAT respectively and in the three analysed depots (MEAT, RPAT and EAT) on day 14, by 11-, 2- and 3-fold ($P < 0.001$) respectively when compared with day 0 (Fig. 4).

Figure 3 Western blotting analysis of protein extracts from MEAT, RPAT and EAT of control (day 0), 7 and 14 days after tumour cells inoculation. Blots were rearranged and most representative images were selected for adiponectin and HSL and β-tubulin proteins (normalised protein). MEAT, mesenteric adipose tissue; RPAT, retroperitoneal adipose tissue; EAT, epididymal adipose tissue; HSL, hormone-sensitive lipase. Values are mean $\pm$ S.E.M. for five animals per group. * $P < 0.05$ vs control (day 0).

In order to evaluate the presence of infiltrated cells in cachexia-affected adipose tissue, the gene expression of CD68, f4/80 and MIF was determined in the different pads during the progression of the disease (Fig. 4). In MEAT and RPAT, an increase in CD68 mRNA levels was evident on day 4 (3- and 2.5-fold), day 7 (8- and 3.7-fold) and day 14 (8.8- and 7.8-fold) respectively ($P < 0.001$). In EAT, changes were detected solely on day 14, with an increase of 1.4-fold ($P < 0.05$). f4/80 mRNA expression increased on day 14 in all adipose tissue depots (MEAT, threefold; RPAT, fourfold; and EAT, fivefold, $P < 0.01$). In addition, in MEAT, the change started already on day 7, with an increase of 10% ($P < 0.01$), when compared with day 0. MIF mRNA expression showed an increase only on day 14 (3.7-fold in MEAT, $P < 0.01$), 3.6-fold and 77% (RPAT and EAT respectively, $P < 0.05$) when compared with day 0.

Discussion

In this study, we demonstrated several morphological and molecular changes in three different adipose tissue depots

of rats during the progression course of cancer cachexia. Most of the alterations were initiated before any detectable morphological disruption, notably through down-regulation of genes related to adipogenesis, metabolism and mature adipocyte function. Changes in plasma adiponectin levels occurred in a parallel manner to the down-regulation of WAT genes, indicating possible disturbances in signalling systems that control WAT homeostasis. In addition, adipose tissue inflammation, a well-characterised symptom of cachexia, was most apparent at the late stage.

Cancer cachexia induces wasting of fat mass that accounts for a large part of the dramatic weight loss observed both in humans (Fouladiun *et al.* 2005, Agustsson *et al.* 2007, Dahlman *et al.* 2010) and in animals (Machado *et al.* 2004, Bing *et al.* 2006, Asp *et al.* 2010), and lipid metabolism is markedly altered (Seelaender *et al.* 1996, Tisdale 2009, Arner 2011). Walker 256-induced cachexia was efficient at eliciting loss of body weight in terms of both absolute and relative weight (percentage of total body mass) of the fat depots, with consequent abnormalities in plasma biochemical profile. While MEAT and RPAT mass decreased to the greatest extent, at least on day 14 (with no change in the intermediary

Table 2 Biochemical and adipokine levels of animal groups during the development of cancer cachexia. Values are mean $\pm$ S.E.M. for seven animals per group

Parameters	Day 0	Day 4	Day 7	Day 10	Day 14
TAG (mg/dl)	65 $\pm$ 18	66 $\pm$ 19	84 $\pm$ 25	85 $\pm$ 18	93 $\pm$ 17*
Glucose (mg/ml)	135 $\pm$ 6	125 $\pm$ 8	125 $\pm$ 11	120 $\pm$ 8*	113 $\pm$ 13*
Protein (μ g/ μ l)	44 $\pm$ 5.0	44 $\pm$ 5.0	44 $\pm$ 7	40 $\pm$ 6	37 $\pm$ 5*
Adiponectin (ng/ml)	9.6 $\pm$ 0.7	14.8 $\pm$ 1.1*	9.6 $\pm$ 1.5	8.0 $\pm$ 1.0	7.3 $\pm$ 0.5*
Leptin (ng/ml)	1.4 $\pm$ 0.2	1.2 $\pm$ 0.2	1.2 $\pm$ 0.3	1.0 $\pm$ 0.3	0.7 $\pm$ 0.3*
Resistin (ng/ml)	5.9 $\pm$ 1.7	6.2 $\pm$ 1.0	17.0 $\pm$ 7.6*	8.1 $\pm$ 2.0	3.9 $\pm$ 1.4

TAG, triacylglycerol. * $P < 0.05$ vs control.

Figure 4 Expression levels of genes involved in mature adipocyte function and in inflammation in different adipose tissue depots during cancer cachexia progression. Real-time PCR analysis of RNA isolated from MEAT, RPAT and EAT of control (day 0), 7 and 14 days after tumour cell inoculation. mRNA levels of target genes were normalised to 18S. MEAT, mesenteric adipose tissue; RPAT, retroperitoneal adipose tissue; EAT, epididymal adipose tissue; MIF, macrophage migration inhibitory factor. Values are mean $\pm$ S.E.M. for seven animals per group. * $P < 0.05$ vs control (day 0).

stage of cachexia – day 7), the mass of EAT did not differ from control. This finding is in agreement with our previous study (Machado *et al.* 2004) and we further demonstrate that reduction in WAT depot weight (absolute and relative) of Walker 256 tumour-bearing rats seems to occur only on the 14th day post-inoculation (after which animals spontaneously die due to cachexia). Adipocyte morphometrical data also changed in the late stage of cachexia, at which time cells from MEAT were found to be larger compared with day 0. By contrast, there was a decrease in the size of the RPAT and EAT cells at the same time point. It is important to note that, at in this point (day 7), there was no difference in absolute and relative WAT weight of all depots. During normal physiological animal growth, there is an increase in the mass of WAT depots, which occurs in parallel to increases in body weight, an aspect well characterised by DiGirolamo *et al.* (1998), who measured adipocyte cell volume. The development of cachexia (days 7–14) affected this pattern, nevertheless in a way that is dependent on the anatomical localisation of the fat

pad: while there was increase in the size of MEAT adipocytes, cachexia caused significant decreases in the size of RPAT and EAT cells (both related to normal tissue growth). Rat MEAT has been shown (DiGirolamo *et al.* 1998) to grow mainly by hypertrophy and normally contains a higher percentage of water than RPAT and EAT. RPAT expansion under physiological conditions occurs predominantly by hyperplasia (DiGirolamo *et al.* 1998), but the results obtained here suggest that cachexia is able to induce a dual effect: hypertrophy on day 7, probably due to normal tissue growth, followed by atrophy on the day 14, probably cachexia induced. EAT showed a reduction in the size of adipocytes in the course of cachexia (day 14), with no changes in relative tissue weight. These regional-specific responses are probably related to the degree of innervation, vascularisation and depot-specific concentration of hormones and cytokines (Pond 1999).

In cancer cachexia, a substantial diminishment in gene transcription of PPAR γ , C/EBP α and SREBP-1c was already demonstrated in WAT from mice (epididymal

depot; Bing *et al.* 2006). We extend this finding by evaluating the expression of adipogenic genes in other visceral depots. PPAR γ mRNA levels showed a conspicuous decrease in all depots evaluated (MEAT, RPAT and EAT) beginning on day 4 following the injection of tumour cells (early-stage cachexia). Ablation of PPAR γ 1 and PPAR γ 2 from the mature adipocytes results in a moderate reduction of adipose mass, which is accompanied by hyperlipidaemia, liver steatosis and protection from high-fat diet-induced increase in adiposity (Jones *et al.* 2005, Heikkinen *et al.* 2007). Given that PPAR γ is an essential transcriptional regulator of adipogenesis and is required for maintenance of mature adipocyte function, including triglyceride synthesis and storage (Yahagi *et al.* 1999), we also evaluated its protein expression. A reduction in protein expression was evident on day 14 in all visceral depots evaluated, indicating that, despite heterogeneity of adipose tissue, PPAR γ is equally disrupted in the late stage of cachexia. In fact, our data demonstrate that in the three depots, there was an extensive reduction in gene as well as in the protein expression of PPAR γ , but the levels of C/EBP α and SREBP-1c only decreased in the retro-peritoneal and epididymal depots. On the other hand, the mesenteric depot showed a striking increase in the expression of SREBP-1c and C/EBP α , gene and protein respectively. This is particularly interesting and sheds some light on the explanation of why the mesenteric adipocytes were not significantly affected by the 'fat-reducing effect' imposed by the tumour, as observed in the other depots. Previous studies have clearly demonstrated the importance of C/EBP α in regulating fat cell formation and function. Furthermore, C/EBP α also triggers the expression of SREBP-1c, which in turn modulates the lipogenic capacity of adipocytes. Several lipogenic enzymes have their expression induced by SREBP-1c including ATP citrate lyase, acetyl-CoA carboxylase and fatty acid synthase. We have not measured the expression of any SREBP-1c target genes in our study, but it is reasonable to expect a reduction in their expression in EAT and RPAT throughout the 14-day protocol and an opposite pattern of expression in MEAT, which could explain a lower lipogenic ability of EAT and RPAT.

The visceral and subcutaneous white fat is recognised as the largest sites of adipokine production in the body. Adipokine expression is highly increased during adipocyte differentiation and tightly controlled by PPAR γ and C/EBP α activity. In this study, the circulating concentration of adiponectin rose almost twofold 4 days after tumour injection, which seems to be particularly influenced by the mesenteric adipose cell contributions, as denoted by the marked enhancement in adiponectin gene expression in this depot, while the other pads presented a negligible change in mRNA and protein expression. Adiponectin expression is modulated by C/EBP α , as consistently reported by others (Park *et al.* 2004). The use of siRNA against C/EBP α in 3T3-L1 cells clearly induces a reduction of adiponectin expression (Vernochet *et al.* 2010). Interestingly, patients with heart failure, who develop cachexia, also present higher levels of

adiponectin (McEntegart *et al.* 2007), and in cancer cachexia patients, this parameter is robustly correlated with body weight loss (Batista Jr *et al.* 2012). On the other hand, leptin secretion, which has been shown to be positively correlated with fat cell size, decreased by 50% 14 days after tumour cell inoculation. These data are consistent with a previous publication from our group, in which Walker 256 carcinosarcoma induced a down-regulation of leptin production (Bertevello & Seelaender 2001, Machado *et al.* 2004). In addition to that, it has been shown that when isolated human adipocytes are incubated with tumour necrosis factor (TNF- α), leptin release is inhibited. TNF- α inhibits leptin production in subcutaneous and omental adipocytes from morbidly obese humans (Fawcett *et al.* 2000).

The presence of adipogenic and morphological defects in visceral WAT in cachexia is further supported by the dramatic reduction in mRNA levels of perilipin. To confirm this alteration, we showed that perilipin protein expression was also reduced on day 14; however, this was just evident in MEAT and RPAT. It is interesting to note that it is the first report, at least as far as we know, showing a down-regulation of perilipin mRNA and protein in fat cells during the development of cancer cachexia in tumour-bearing rats and that, despite the heterogeneity herein related, this reduction is similar in visceral WAT depots. We also evaluated HSL protein expression in different experimental time points and WAT depots. Recently, it has been shown that lipolysis plays an instrumental role in the development of cancer cachexia (Das *et al.* 2011). Cachexia induced by Walker tumour elicited an increase in HSL protein expression at day 14 only in MEAT depots, indicating that HSL seems to be disrupted in the late stage of cachexia in a depot specific manner. Perilipin is the most abundant protein on the surfaces of adipocyte lipid droplets, being involved in the regulation of basal and hormonally stimulated lipolysis (Brasaemle 2007). In times of energy deficit, perilipin facilitates maximal lipolysis by providing a dynamic scaffold to coordinate access of HSL and adipose triglyceride lipase to the lipid droplet in a manner that is responsive to the metabolic status of the adipocyte (Meijer *et al.* 2011). However, the exact role of perilipin and HSL in adipose tissue dysfunction in the setting of cachexia requires further investigation.

Haptoglobin is an acute-phase reactant protein secreted into the plasma participating in many biological processes including immune regulation, inflammation and malignancy. The present results demonstrate a massive increment in haptoglobin gene expression in the mesenteric depot, yet less prominent in the other depots. We hypothesise that the reason for such an increase might be related with the participation of PPAR γ and TNF- α as upstream mediators of haptoglobin production. Cancer cachexia induces a scenario with elevated levels of pro-inflammatory cytokines (Argiles *et al.* 2009, Lira *et al.* 2012, Seelaender *et al.* 2012), including TNF- α , which in turn could inhibit PPAR γ expression and activity but stimulate haptoglobin expression. In this regard, increased levels of gene and protein expression of TNF- α in MEAT of

Walker 256 tumour-bearing rats is well characterised (Machado *et al.* 2004, Lira *et al.* 2012). Confirmation of a lower PPAR γ activity in our animals is granted by the observation of reduction in leptin and perilipin expression, as well as a decrease in perilipin protein content.

Appearance of infiltrated cells (leucocytes) and/or mediators of inflammation has been postulated to contribute to the pathogenesis in the development of impaired adipose tissue function and ectopic fat deposition (Bluher 2009); however, it is not clear how these events take place during the development of WAT dysfunction. Therefore, we evaluated gene expression of specific macrophage phenotype (f4/80 and CD68) and survival (MIF) markers. CD68, a classical macrophage marker, showed up-regulation in all visceral depots on day 14; however, this change began on day 4 in MEAT and RPAT, suggesting an increase in mononuclear cells in adipose tissue during the initial periods of the development of cancer cachexia. These data corroborate our previous study (Machado *et al.* 2004). However, it is important to note that CD68 is not only restricted to the macrophage lineage but also expressed in fibroblasts, and changes in the adipose tissue matrix environment (fibrosis) may reflect increases in this cell type. To further elucidate the presence of infiltrated macrophages during the development of cancer cachexia, we also evaluated f4/80 gene expression, which was similarly increased in all visceral depots on day 14. Another evaluated gene, MIF, which has an important function as a pleiotropic protein, participating in inflammatory and immune responses as well as in delayed hypersensitivity and various macrophage functions, including phagocytosis, spreading and tumoricidal activity, showed an increase only on day 14 in MEAT and RPAT and, on the other hand, was decreased in EAT. On the other hand, the possible relationship between infiltrated cells and impairment of adipose tissue function needs additional investigations.

In summary, our study demonstrates pronounced molecular and depot-specific alterations of WAT in tumour-bearing animals, which indicate a time-dependent effect of cachexia on adipose tissue. Regarding molecular disruption, down-regulation of the key adipogenic factors, as well as repression of lipogenic factors, suggests impairment in the formation and lipid-storing capacity of adipose tissue, which occurs very early during the development of cancer cachexia. Finally, classical markers of inflammation and macrophage infiltration in WAT were only evident in the late-stage cachexia, at the same time that morphological and plasma biochemical disturbance were observed, indicating that several molecular disruptions on WAT occur before establishment of any detectable inflammatory parameter.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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