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Adipose tissue-derived factors as potential biomarkers in cachectic cancer patients

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ABSTRACT

Cachexia, a paraneoplastic syndrome markedly associated with worsened prognosis in cancer patients, provokes profound wasting of both lean and adipose mass in an association with a state of metabolic "chaos". The white adipose tissue responds to cachexia with marked local inflammation and may be thus a relevant contributor to systemic inflammation. To address this hypothesis we examined the correlation between tissue expression of adipokines and plasma concentration in cachectic and stable weight patients with or without cancer. Adiponectin and liver-derived CRP concentration were significantly higher in the cachectic groups when compared with stable weight patients ($P < 0.01$). The concentration of plasma IL-6 was higher (11.4-fold) in the cancer cachectic group when compared with weight-stable controls, and presented a significant correlation with the presence of cancer ($P < 0.001$). A marked increase (5-fold) in IL-6 as a result of the interaction between the presence of cachexia and the presence of tumour was observed in the subcutaneous tissue of the patients, yet not in the visceral depot. Plasma adiponectin levels were higher in cachectic cancer patients, compared with stable weight cancer patients individually matched by age, sex, and BMI, and the subcutaneous depot was found to be the main contributing tissue, rather than the visceral pad. Based on the results we concluded that the subcutaneous adipose tissue is associated with plasma changes that may function as markers of cachexia.

1. Introduction

Cachexia, a wasting syndrome is associated with 22–40% of cancer deaths [1,2]. Progressive cachexia represents an independent prognostic factor concerning the response to antineoplastic therapy and survival [3], and has been recently defined as "a multifactor syndrome characterized by ongoing loss of skeletal muscle mass (with or without loss of fat mass) that cannot be fully reversed by conventional nutritional support and leads to progressive functional impairment" [4]. Although muscle wasting has been the main focus of cachexia-related research [5,6], studies show that fat loss occurs more rapidly and more precociously than the reduction of lean mass in cancer cachexia [7,8], and may extend up to 80% within a very short interval, especially in the immediate period preceding death [9].

The underlying molecular basis of cachexia is still poorly understood, yet the perspective of the syndrome as a chronic inflammatory state, in which the host's reaction to the presence of the tumour seems to be the main causal agent, is gaining crescent acceptance [10–12]. Inflammation may be a result of the production of mediators deriving both from the tumour and from the host's tissues, among which inflammatory cytokines appear as major contributors [6].

We have shown [13–15] that the white adipose tissue (WAT) actively expresses and secretes a plethora of pro-inflammatory factors in a rodent model of cancer cachexia and may thus be considered an important subscriber to the characteristic systemic inflammation. Furthermore, we hereby propose that factors deriving from this tissue may be adopted as markers for the diagnosis and grading of the syndrome, based on the aforementioned animal studies and on the results we now present with cancer patients.

WAT has been shown to largely subsidise systemic inflammation in a variety of diseases, as it has, over the last decades, been recognised to be a major endocrine organ, capable of actively synthesising and secreting a plethora of humoral factors, the adipokines, among which leptin, adiponectin, TNF-α, IL-6, IL-10, plasminogen activator inhibitor-1 and visfatin; all of which act locally in an autocrine/paracrine manner and/or as endocrine signals in the regulation of appetite, energy expenditure and a range of physiological processes including insulin sensitivity and the inflam-

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matory response [16]. Increased production of lipolytic factors by the adipose tissue and the tumour, such as interleukin 6 (IL-6), tumour necrosis factor α (TNF-α) or zinc-α2 glycoprotein (ZAG) contributes to the disrupted lipid metabolism and increased lipolysis in cancer cachexia [5,17]. Therefore, the white adipose tissue is both a victim and a sponsor of cachexia-related systemic inflammation.

In this study we present results with gastrointestinal cancer patients that may support the adoption of WAT-derived factors as markers of cachexia. The recently proposed grading of cachexia [2], envisaging three stages, namely; pre-cachexia, cachexia, and refractory cachexia, signals the necessity of establishment of tools allowing for unequivocal diagnosis aiming at precise intervention, according to the patient's actual disease stage and prevention of its aggravation.

2. Patients and methods

2.1. Patients

All patients were recruited between November 2008 and July 2011 at the ambulatory surgery division from the University Hospital of the University of São Paulo ($n = 235$). The inclusion criteria were; 1 – no prior anticancer treatment, 2 – not having clinical evidence of gastrointestinal obstruction, 3 – fitting into the logistical scheme for scientific studies before operation or preoperative treatment and 4 – willingness to participate ($n = 61$). The exclusion criteria were liver failure, renal failure, AIDS, inflammatory diseases of the bowel and autoimmune disorders. The study was approved by the Regional Ethics Committee (CEP-HU/USP 752/07, CAAE 0031.0.198.019-07). The investigation was explained in detail to each patient and a written informed consent was obtained. The patients were divided into four groups based on diagnosis after surgery. The cachectic groups were subdivided into two groups: cancer cachexia ($n = 31$), and control (non-cancer) cachexia ($n = 8$). Cancer cachexia was defined as prediagnosed gastrointestinal cancer with no evidence of gastrointestinal obstruction or anorexia with nausea and/or stomach pain, in combination with unintentional weight loss of >5% of the habitual weight during the last 3 months or >10% weight loss during the last 6 months. The same criteria were adopted to classify control cachexia, excluding cancer diagnosis. The weight-stable groups (no important weight change during last year and BMI <25) were divided into weight-stable cancer ($n = 12$) and weight-stable control (non-cancer) patients (control, $n = 10$). In the cancer groups, the tumour primary location was: colon ($n = 14$), stomach ($n = 13$), pancreas ($n = 3$), rectum ($n = 2$), and others ($n = 11$). The cachectic control group was composed by patients with: cirrhosis ($n = 3$), chronic pancreatitis ($n = 3$), and gastric or duodenal ulcers ($n = 2$). The weight-stable control group included patients undergoing surgery for incisional ($n = 7$), inguinal ($n = 2$) and umbilical ($n = 1$) hernia. The study was designed as "intention to compare"; therefore, all subjects were kept in the analyses despite a few missing values of the measurements. The remaining 174 patients were excluded because (a) although pre-diagnosed with gastrointestinal cancer they did not have malignancy according to final histological evaluations ($n = 41$) or (b) could not provide adequate amounts of adipose tissue, at least 0.9 g or (c) could not provide, for the same patient, samples from subcutaneous and omental adipose tissue depots and samples related with inflammatory and biochemical parameters, for a complete investigation. Table 1 presents the general characteristics of patients in each group.

2.2. Clinical parameters assessment

Height and weight were determined and approximately 10 mL of blood were collected after an overnight fasting, within the venous access procedure for anaesthesia during the surgery, allowing the measurement of plasma lipids, glucose, serum urea, and hemoglobin. Plasma and serum samples were immediately frozen at −80 °C until further analysis. Tumour staging was determined postoperatively according to the guidelines of the UICC TNM [18]. Cachexia staging, anorexia and inflammation were assessed by the Cachexia score [19].

2.3. Adipose tissue biopsies

Approximately 1 g of subcutaneous and omental white adipose tissue (by approximate anatomical site), were collected at 5 min intervals, similarly to described by [17]. Tissue samples were rapidly rinsed in saline, frozen in liquid nitrogen, and kept at −80 °C for further analyses. This procedure presented a minimal degree of risk, and did not interfere with surgery routine.

2.4. Plasma measurements

ELISA kits were employed for the measurements of TNF-α and IL6 (CytoSet™, Invitrogen); CRP (cat.# HCVD2-67BK-01), Adiponectin (cat.# HADK1-61K-A), Leptin (cat.#HENDO-65K-01), IL10 (cat.# MPXHCYTO-60K-02), (Genese Produtos Diagnósticos Ltda., Brasil) and Multiplex kits, for Luminex platform (R&D Systems, USA). Adipokine and cytokines concentration was determined in triplicate, and the mean was used for analysis. The inter-assay CV over all ranges was lower than 12%.

2.5. Gene expression analysis

Total RNA of the samples was isolated with TriPure Isolation Reagent (Roche®) following the recommendations of manufacturer and total RNA concentrations quantified by spectrophotometry (Nanodrop ND-1000). Complementary DNA synthesis was carried out with 13 μl assay mix containing 3 μg total RNA, 10U RNAse inhibitor, 2 μl random primers, 2 μl dNTP (10 nmol), 2 μl dithiothreitol, 10U M-MLV reverse transcriptase and 4 μl of 10× reaction buffer (100 mM TRIS–HCl, 500 mM KCl; 150 nM $MgCl_2$ in nuclease free water) (Invitrogen). The mRNA expression of the following genes was determined: IL-6 (NM_000600.3, Forward 5′TAC CCC CAG GAG AAG ATT CC3′, Reverse 5′AGG TTG TTT TCT GCC AGT GC3′), Adiponectin (NM_001177800.1, Forward 5′ATG ACC AGG AAA CCA CGA CT3′, Reverse 5′CAC CGA TGT CTC CCT TAG GA3′), TNF-α (NM_000594.2, Forward 5′ CTC TCT CCC CTG GAA AGG AC 3′, Reverse 5′ATC ACT CCA AAG TGC AGCA G3′), IL-10 (NM_000572.2, Forward 5′CCA AGC TGA GAA CCA AGA CC 3′, Reverse 5′AAG GCA TTC TTC ACC TGC TC 3′), and leptin (NM_000230.2, Forward 5′GGA GGG CAA GGG CCA TGC TG′3, Reverse 5CT GGC CAC AGC ACCA GCC TC′3). Five μL of cDNA (25 ng) were mixed with 2x SYBR Green PCR master mix (Applied Biosystems) and primers (Invitrogen). Quantitative real-time PCR was performed with an ABI 7300 Real Time Systems (Applied Biosystems). The mRNA levels were determined by comparative Ct method for each sample. A ΔCt value was obtained by subtracting 18S values from those of the gene of interest. The average ΔCt value of the control group was then subtracted from the sample to derive a $\Delta^{-\Delta Ct}$ value. The expression of each gene was evaluated by $2\text{-}(\Delta^{-\Delta Ct})$.

2.6. Statistical analysis

The statistical analysis was performed with the commercially available statistical package from SigmaStat (version 3.1, SigmaStat, SYSTAT, Point Richmond, CA). Data were expressed as means ± SE and analysed by 2-way ANOVA of $2 * 2$ design (cancer/non-cancer-control vs. stable weight/cachexia). Data were parti-