Impaired Glucocorticoid Synthesis in Cancer Cachexia-Anorexia Syndrome in an Experimental Model

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Abstract

The adrenocorticotropic hormone (ACTH) acts in the metabolism regulation through the control of adrenal synthesis of glucocorticoids; these hormones have important physiological and anti-inflammatory actions, during an injury or illness. In consumptive syndromes, such as neoplastic cachexia, corticotropic axis is a critical component of the adaptive neuroendocrine responses. To study the morphological and physiological alterations of the pituitary and adrenal glands during cancer development, we used an experimental model of tumor-induced cachexia and anorexia by inoculating Walker-256 cells in rats which experienced two different stages of cancer development. After a functional test with ACTH1-24 to evaluate the adrenal function, the tumor was dissected and the pituitary and adrenal glands were removed for histopathological and immunohistochemical studies. The results showed a marked body weight loss early on day 5 of tumor development with 12% cachexia index. Morphological changes such as cell growth in the adrenal cortex and increased size of the corticotrophin cells in the pituitary anterior lobe were also evidenced with few differences at day 12. Despite the signs of HPA axis activation such as adrenal and pituitary hyperplasia and hypertrophy, and the immuno reactivity for ACTH, the functional test with ACTH1-24 and the adenalin immunostaining for 3βHSD pointed to adrenal insufficiency. Both hyperplasia and hypertrophy of pituitary and adrenal glands are morphological changes resulting from the activation of corticotrophic axis in response to stress and occurred early in the development of neoplastic cachexia in Walker-256 tumor bearing rats. However, since this response was associated with insufficient secretion of corticosterone and low levels of the steroidogenic enzyme 3βHSD in the advanced cancer, we conclude that the development of the cachexia anorexia syndrome induces HPA axis hyperactivity followed by primary adrenal insufficiency.

ABBREVIATIONS

HPA: Hypothalamus-Pituitary-Adrenal; CRH: Corticotrophin Releasing Hormone; ACTH: Adrenocorticotropic Hormone; MC2R: type 2 Receptor for Melanocortin; 3βHSD: 3-Beta-Hydroxysteroid-Dehydrogenase

INTRODUCTION

The cachexia-anorexia syndrome is a multifactorial syndrome that results from several abnormalities of metabolism, like muscle proteolysis, lipolysis in adipose tissue, increased fat mobilization, hepatic neoglucogenesis and peripheral glucose resistance, accompanied by profound weakness, anorexia and anemia [1]. This syndrome occurs in chronic processes including cancer, AIDS, diabetes mellitus and severe rheumatoid arthritis [2]. Although cachexia always includes a component of reduction in food intake, it differs from malnutrition and depletion by the fact that there are also metabolic alterations and inflammatory mediators involved such as peptide hormones, neurotransmitters and cytokines [3,4]. This means that the loss of body mass can occur more quickly than expected simply by reducing of food intake of equal magnitude, and also the loss can be higher for non-fatty tissues [5]. The experimental model of cachexia in rats, induced by the Walker tumor, is widely used for the understanding of the neoplastic cachexia. The Walker-256 carcinosarcoma was developed from a spontaneous carcinoma originated in the mammary gland of albino pregnant rats to establish the tumor cell line LLC-WRC 256. Since then, these cells has been kept largely in the form of carcinosarcoma highly invasive and undifferentiated, being an important resource in basic research [6]. Various aspects relating to their biological behavior as their ability to induce a rapidly state of neoplastic cachexia and their biochemical alterations have been studied [7].

The hypothalamus-pituitary-adrenal axis, known as HPA axis, and the sympatho-adrenomedullary axis are the primary systems that are responsible for the homeostasis during stress, and the adrenal gland is an essential organ that is common to both systems [8]. The adrenal gland participates in the acute and chronic response to stress with a medullar response with secretion of adrenaline and noradrenaline under the influence of the autonomic nervous system and with a cortical response...
with the glucocorticoid secretion under the influence of the HPA axis [9]. The corticotrophin releasing hormone (CRH) is the main control of the HPA axis coming from the paraventricular nuclei of the hypothalamus, and stimulating the adrenocorticotropic hormone (ACTH) secretion from the pituitary gland [10]. The target organ of the HPA axis is the adrenal gland, whose cortical layer contains a number of enzymes responsible for the steroidogenesis, activated by the ACTH binding to its receptor MC2R (type 2 receptor for melanocortin or corticotropin receptor) [11]. ACTH controls both the cell proliferation and the steroidogenic activity of the gland. Its suppression leads to the adrenal cortex atrophy while ACTH excess causes hyperplasia of the fasciculata and reticularis zones.

The interaction between HPA axis and cancer development is not fully understood and raises clinical concerns [12,13]. Corticosteroids have potential usefulness in the treatment of anorexia-cachexia syndrome in cancer; on the other hand, glucocorticoid excess can induce cachexia and might contribute to cancer progression [14]. Experimental models are valuable tools to address such questions. We have demonstrated that stimulated steroid secretion was significantly decreased in the model of neoplastic cachexia induced by Walker tumor, by measuring the plasma corticosterone response to submaximal doses of ACTH. To clarify the pathophysiological basis of this suppression, we followed the growth of Walker tumor until the last stage of cancer development and noticed that this was characterized by adrenal hyporeponsiveness. The purpose of this study was to assess the effects of the tumor-induced cachexia syndrome on the adrenal and pituitary glands. Hence, the morphofunctional integrity of the main endocrine components of the HPA axis was examined in both short-term and long-term tumor development.

MATERIALS AND METHODS

Animals and cell inoculation

Male Wistar rats weighting 200-250 g, kept at temperature of 23.2°C, light/dark cycle of 12 h and with free access to water and standard rodent chow diet were used. Pair feeding control was not performed since no difference was observed in our previous analysis of adrenal and pituitary glands from control animals.

Walker-256 cells, cultivated intraperitoneally in rats, were collected, centrifuged, resuspended in phosphate-buffered saline (PBS; 16.5mmol/L of phosphate, 137mmol/L of NaCl and 7mmol/L of KCl), pH 7.4, and the cell viability was evaluated using the method of trypan blue exclusion. Wistar rats were inoculated subcutaneously with 8.0 x 10⁹ viable tumor cells/animal in the right rear flank. Control rats were inoculated with PBS in the same inoculation site. The protocols of the experiments were approved by the Ethics Committee for Animal experimentation of the State University of Londrina.

Experimental design

Two cohorts of rats were used in this study. The first cohort was evaluated for functional activity of the adrenal cortex with the ACTH synthetic challenge test. Both control and tumor group (n=8) were followed until the 12th day of tumor evolution. Then, animals were anesthetized i.p. with a solution of xylazine (19mg/kg) and ketamine (90 mg/kg). After 5 minutes, blood was collected in heparinized microtube for the measurement of basal corticosterone. For the adrenal stimulus test, synthetic ACTH₁-24 (Synacthen®, Novartis Pharma) was administered at a concentration of 42 pmol/Kg i.p. and the blood was collected in heparinized microtube after 15 minutes, followed by cervical dislocation. Plasma corticosterone levels were assessed in duplicated samples by immunoassay, according to the recommendations of the supplier (corticosterone EIA Kit, Cayman Chemical).

In the second cohort of rats, the animals were divided into the three following groups (n=10): rats with Walker-256 tumor sacrificed on days 5 and 12 after implantation of tumor (tumor-bearing rats) and rats without tumor (control rats). Those animals were subjected to the study of weight changes and histopathology analyses of adrenal and pituitary tissues.

Characterization of cachexia and evaluation of related parameters

For the second cohort of rats, on the 5th and 12nd days, rats were weighed, euthanized and their adrenal, pituitary and tumor mass were carefully dissected and weighed to calculate the percentage of body mass loss (cachexia index), expressed in %. For this evaluation, we used the following parameters: initial weight (iw), final weight in the rat with tumor (fw), tumor mass (tm), and weight gain in control rat (wgc), accordingly to the formula:

\[
\% \text{ loss of body mass} = \frac{\text{iw} - \text{fw} + \text{tm} + \text{wgc}}{\text{miw} + \text{wgc}} \times 100
\]

The host weight was defined as the total body weight of the animal subtracting the tumor weight. The tumor volume was measured with a caliper at different days of tumor development until the sacrifice day; for this purpose, we used a volume ellipsoid formula (length x depth x width x 0.5233) [15].

Histopathological study

The pituitary and adrenals were fixed in buffered 10% formaldehyde and then paraffin embedded for conventional morphology study. Longitudinal pituitary and adrenal sections were stained with haematoxylin and eosin stain. The morphological and morphometrical analysis was done on 4 μm sections with light microscopy. The morphometrical analysis of adrenal cortex was performed using a millimeter scale in the eyepiece of a Nikon microscope (E-200, Tokio, Japan) with 10 x objective.

For immunohistochemical reaction, paraffin embedded adrenal and pituitary sections were dewaxed, hydrated and heat treated in 1 mM EDTA buffer for antigenic unmasking on microwave at 95.8°C for 20 min. Sections were incubated overnight at room temperature with rabbit polyclonal anti-ACTH (Dbiosys, Califórnia, USA; 1:250) to identify the corticothrophs cells in the anterior lobe of the pituitary and the adrenal sections with rabbit polyclonal anti-3βHSD (3-beta-hydroxysteroid-dehydrogenase) (Bioss, London, UK; 1:100) to identify the immunoexpression of the enzyme in the adrenal cortex cells. Immunohistochemical staining was done using the polymer peroxidase based method, followed by development with diaminobenzidine. A methodical negative control went through
the first step of the procedure (incubation with vehicle instead of the primary antibody).

**Digital computer-assisted immunohistochemistry quantification**

Histological slides were analyzed under the optic microscope to identify areas that best represented 3βHSD or ACTH immunostaining, whereas brownish-color was considered to be evidence of a positive expression. From each sample, photomicrographs of 800x600 pixels were obtained from x4 magnification fields, using a Samsung camera (three images/slice), adapted in the optic microscope. Digitally acquired images were analyzed with Image J 1.44 software for Windows, using the threshold tool with color-based selection for positive staining. The number of pixels included in the selected color range was divided by the total number of pixels in each field. Four slices per sample of tissue were analyzed and data were averaged. Results were expressed by the relation between the immunopositive area fractions per total area fraction (Supplementary Figure 1).

**Statistical procedures**

One-way ANOVA was used to compare variables at the end of the experiments. When the overall ANOVA was significant, pairwise comparisons were performed using Tukey’s method. The t-test was used to compare two groups and the Pearson test was used for evaluate the correlation between two variables. Statistical analyses were performed using GraphPad Prism software, version 4.0 (GraphPad Software, San Diego, USA) and Statistical Package for the Social Sciences (SPSS) Version 21.0 for Windows (SPSS, Chicago, USA). P<0.05 was considered significant in all tests.

**RESULTS**

The evaluation of the basal plasma levels of corticosterone on day 12 showed no significant difference between the control and tumor groups. However, after the adrenal cortex stimulation test, we observed a significant reduction in corticosterone secretion in tumor bearing rats of approximately 24% (Figure 1).

Rats weighted approximately 220 g (211-227) before tumor inoculation. While the control group experienced an increase of 9% and 21% of body weight on days 5 and 12, the tumor bearing rats had 2±0.34% and 19±0.2% of gain when compared with the first day. This expressive gain of body weight in tumor bearing rats on the 12th day was due to the presence of the tumor mass. In fact, there was about 10% decrease in the host body weight if the tumor mass was subtracted from the whole body (Figure 2). Similarly to the lost weight, there was a reduction of approximately 37% in food intake of tumor bearing rats when compared with controls animals, considering the mean of the five last days (data not shown). In studied tumor bearing rats, we observed a progressive increase in both tumor volume and in the tumor mass (Figure 3 A, B), as expected by its malignance; its weight reached approximately 5±0.6 g on day 5 and 23±1.6 g on day 12 after the cell inoculation. Tumor bearing rats but not control rats developed anorexia and lost body mass at an early stage of tumor development, from the 5th day, the loss of body mass resulted in significant and progressive cachexia index higher than 10 % (Figure 3C).

**Figure 1** Glucocorticoid function of the adrenal cortex. A. Basal plasma corticosterone level; B. Plasma corticosterone after the stimulation test with synthetic ACTH 1-24 in control and tumor bearing rats at day 12. Each bar represents mean ± SEM; (n=4/group). **P < 0.01(t-test).

**Figure 2** Time course of change in body weight, expressed as % of initial weight in control and tumor bearing animals. The host weight was calculated by subtracting the tumor weight from the total body weight. Each bar represents mean ± SEM; (n=10/group).*P <0.05 and **P < 0.01 versus control (protected t-test).

**Figure 3** Tumor and cachexia progression. A. Time course of growing palpable tumor in tumor bearing rats during the study; B. Tumor weight and C. Cachexia index in tumor bearing rats on days 5 and 12 after implantation of tumor. Each bar represents mean ± SEM (n=10/group). **P < 0.01 (t-test).
In our study, weight and morphology of adrenal and pituitary glands were evaluated in both control and tumor groups. Absolute adrenal weight in tumor bearing rats on days 5 and 12 was increased by 73 and 50 % respectively, compared with the control rats (Figure 4 A). The adrenal weight relative to the body weight was also increased in tumor bearing rats (p<0.05) (Figure 4 B). By the analysis of the adrenals gland at the optic microscope, cortex and medulla were evidenced as two distinct regions, without any tumor metastasis that could increase the glandular weight (Supplementary Figure 1). The measurement of cortex width showed enlargement by 34.2 ± 1.8% and 22.1 ± 0.6% in tumor bearing rats on days 5 and 12 respectively, compared with control (Figure 4 C). This increase was mainly due to the hypertrophy of the cells in the fascicular zone, the main site of glucocorticoids synthesis. The pituitary weight in tumor bearing rats on days 5 and 12 was increased by 55.1±1.3% and 47.2±1.6%, compared with control (Figure 5 A). We also observed increase in the pituitary weight relative to the body weight in tumor bearing rats on days 5 and 12 in comparison with the control rats (Figure 5 B), but no difference in the pituitary architecture was evidenced by the optic microscopic analysis after HE staining (data not shown). On calculating Pearson’s correlation coefficient, the comparison between relative adrenal and pituitary weights resulted in significant moderate positive correlation (Figure 6A). Regarding the other morphometrical parameters, there was no correlation between them and the development of tumor or cachexia, excepting the pituitary weight that was positively correlated to the tumor weight (Figure 6B).

The 3βHSD enzyme plays a crucial role in the biosynthesis of all classes of hormonal steroids. Immunohistochemical studies in the rat reveal its localization in the three layers of the adrenal cortex, with a cytoplasmatic expression as evidenced by our results (Supplementary Figure 2). The quantitative analysis of the immunoeexpression for the enzyme 3βHSD has been well correlated with the glucocorticoid synthesis and the functional testing [16,17]. The immunoeexpression of the enzyme 3βHSD in the adrenal cortex was reduced in adrenal glands from tumor bearing rats. The stained area to total tissue area ratio in control rats and tumor bearing rats on days 5 and 12 after the tumor implantation was respectively 2.91±0.57, 0.56±0.11 and 0.58±0.16 (Figure 7). Immunocytochemical reactions for

Figure 4 Adrenal morphometrical parameters. A. Adrenal weight; B. Relative adrenal weight; C. Cortical width in control and tumor bearing rats on days 5 and 12 after implantation of tumor. Each bar represents mean ± SEM (n=10/group)* P < 0.05; ANOVA. There was no significant difference between days 5 and 12 in the three parameters analyzed.

Figure 5 Pituitary morphometrical parameters. A. Pituitary weight and B. Relative pituitary weight in control and tumor bearing rats on days 5 and 12 after implantation of tumor. Each bar represents mean ± SEM. (n=10/group) *P < 0.05 ANOVA. There was no significant difference between days 5 and 12 in two parameters analyzed.

Figure 6 Comparison between tumor, pituitary and adrenal weights. A. Correlation between the pituitary weight and the tumor weight (r=0.64, P=0.002; Pearson correlation test). B. Correlation between the relative pituitary and adrenal weights in both control and tumor bearing rats (r=0.53, P=0.0003; Pearson correlation test).
ACTH revealed numerous immunoreactive cells in the anterior lobe of the pituitary forming groups and single cells, diffusely distributed throughout the gland (Supplementary Figure 3A). The intermediate lobe presented strong staining but it was excluded from the selected area during digital computer-assisted immunohistochemistry quantification, because intermediate cells have diverse functional and morphological features. In shape, the corticotrophs were usually triangular or stellate; rounded cells were also seen with a cytoplasmatic ACTH immunostaining in the anterior lobe (Supplementary Figure 3B). The immunohistochemistry study of the pituitary gland demonstrated increase by 53% in the ACTH immunoreactivity in the anterior lobe of the pituitary on day 5, which was sustained on day 12 (Figure 8).

In order to evaluate the HPA response in cachexia tumor induced, we compared histopathological, morphometrical, functional and immunohistochemical data. There was no statistically significant correlation between the immunoexpression of the ACTH at the pituitary and the 3βHSD at the adrenal gland or between each of them and the body weight loss.

**DISCUSSION**

The loss of weight and body mass index characterize the state of cachexia, which may be mediated by an increase in circulating levels of inflammatory cytokines as an adaptative response to the aggression triggered by growth of the tumor mass. According to this, the tumor volume increased significantly in our experiment in parallel to the development of cachexia-anorexia syndrome in Walker tumor bearing rats. It is known that the development and progression of cancer cells can acutely induce the production of cytokines which interact with immune and inflammatory cells activating the HPA [18] but the effect of the disease progression on the endocrine glands have never been studied. In the present study, the morphofunctional evaluation of adrenal and pituitary glands suggested that the synthesis of glucocorticoids suffers impairment during the modulation of the HPA axis.

In our study we observed an increase in the morphometrical parameters of the adrenal and pituitary gland in tumor-bearing rats compared with control. Both endocrine glands suffer adaptation process, like hypertrophy and hyperplasia, during...
disturbances of homeostasis under modulation of the HPA axis. The first effect induced by ACTH is hypersecretion of the adrenal cells and hyperplasia only occurs if the stimulus is maintained long enough [19]. Such data could explain the increase in adrenal weight and hypertrophy probably occurring around 24 h after ACTH stimulation and thereafter. A moderate correlation between the relative weight of the adrenal and pituitary gland observed in our study, demonstrates that the stimulation of the HPA axis had occurred in the same manner on the two glands.

We used immunohistochemistry to better characterize the adrenal and pituitary glands hormone production. Quantitative immunohistochemistry methods have been a good approach to study peptide content, and these data showing good correlation with absolute concentrations of the peptides like ACTH, as measured by RIA (radioimmunoassay) in glands extracts [20]. The regulation of ACTH synthesis and its secretion is a complex process; corticotrphin releasing hormone (CRH) is the most important hypothalamic factor that stimulate pituitary function and hypertrophy, and also induces feeding inhibition. Thus, the increase in the ACTH immunexpression observed in our study is possibly due to the CRH hypersecretion by hypothalamic neurons [21]. High CRH levels can contribute to the decrease of food ingestion by the animals inoculated with tumor [22].

Despite the signs of HPA axis activation according to adrenal and pituitary morphometry and the immunoreactivity for ACTH, we have observed a significant decrease in the corticosterone secretion after the synthetic ACTH stimulation test and a decrease in the 3βHSD enzyme immunexpression indicating impaired activity of steroidogenic cells mainly in the fascicular layer of the adrenal gland. Some studies in cancer states demonstrated progressive reduction in plasma levels of corticosterone in tumor-bearing mice, indicating functional impairment of adrenal glands [23]. Failure to produce adequate levels of glucocorticoids can occur due to a disorder of the adrenal glands themselves (primary adrenal insufficiency) or to the inadequate secretion of ACTH or CRH by the pituitary gland or hypothalamus (secondary adrenal insufficiency) [24]. The hypocortisolism is a common disorder observed during severe illness because many factors can disrupt HPA function, including the high level of inflammatory cytokines. Necrosis or hemorrhage of the hypothalamus or the pituitary gland, observed in severe conditions like sepsis, are related with secondary adrenal insufficiency [25]. Circulating proinflammatory mediators, such as TNF-α, may block the ACTH release and the local expression of TNF-α and IL-1β may interfere with CRH and ACTH synthesis [26]. These are improbable conditions in our study because the pituitary gland demonstrated signs of increased synthesis of ACTH.

While circulating proinflammatory cytokines such as TNF-α may inhibit ACTH-induced cortisol release, the neutrophil-derived corticostatins such as α-defensins can compete with ACTH for binding on its cognate receptor, resulting in adrenal insufficiency in inflammatory states [27]. Moreover, low HDL-cholesterol levels and also adrenal hypoperfusion had been associated with a low cortisol response to the ACTH test in patients with sepsis [28]. Corticosterone synthesis is dependent on an adequate supply of cholesterol to the adrenal cortex and high density lipoprotein (HDL) appears to be the most important source for its production in rodents. HDL cell uptake occurs via a scavenger receptor (SR-BI) [29]; previous studies observed a decreased in HDL-cholesterol in both experimental animals and human subjects with cancer [30]. Our previous results pointed to a marginally significant tendency towards HDL-cholesterol reduction in Walker tumor bearing rats [31]. These conditions could explain the physiopathology of Walker-induced tumor cachexia with ACTH hypersecretion and corticosterone hyposecretion. Moreover, in some aggressive types of cancer there is the possibility of injury by direct adrenal metastasis impairing adrenal function [32], but it was not observed in our experiments.

CONCLUSION

Morphometrical changes of the pituitary and adrenal glands found in our study are related to the HPA activation due to the development of cachexia-anorexia syndrome. However, the stress response to the homeostasis disturbances in the beginning of the disease is not a demonstration of sustained hypercorticism in cancer cachexia. Such changes were accompanied by anorexia, weight loss and fatigue in advanced stage of cancer, which is consistent with primary adrenal insufficiency according to the functional test and the immunohistochimical analysis. Adrenocortical exhaustion following initial hyperactivity could be induced by several conditions including tumoral proinflammatory cytokines, adrenal hypoperfusion and low level of cholesterol as a precursor for the biosynthesis of steroid hormones. However, the mechanisms for impaired steroidogenesis are not completely understood and will require further research. Understanding the activity of the HPA axis in this condition may indicate possible targets for pharmacological intervention in the treatment of this syndrome related to cancer.

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Supplementary Figure 1 Representative images of adrenal cortex used to evaluate the cortical width in control and tumor bearing rats at the indicated time after inoculation (HE, Magnification 4x). C, cortex; M, medulla.

Supplementary Figure 2 Representative images of immunohistochemical staining of the 3βHSD (brown area) in the adrenal gland from a control rat. A. Original magnification x 4, B. Inset: view of the cortex (x10); polymer peroxidase-based method.

Supplementary Figure 3 Representative images of immunohistochemical staining of the ACTH (brown area) in the pituitary gland from a tumor bearing rat (day 5). A. Original magnification x 4, AL, anterior lobe, IL, intermediate lobe, PL, posterior lobe; B. View of the anterior lobe (original magnification x100), arrows indicating the corticotrophic cells; polymer peroxidase-based method.