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Stress hormones concentrations in the normal microenvironment predict risk for chemically induced cancer in rats

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ABSTRACT

Evidence show that stress hormones can influence cancer progression, but its role in carcinogenesis is poorly understood. In this study, we used a new method based on oral carcinogenesis model in rats to test the hypothesis that physiological levels of stress hormones in the normal tissue microenvironment would have significant predictive value for chemically induced cancer occurrence. Male Wistar rats were submitted to a tongue biopsy for measuring not-stress induced levels of norepinephrine, corticosterone, adrenocorticotropic hormone (ACTH) and brain-derived neurotrophic factor (BDNF) in the tissue before carcinogenic induction. Rats were treated with the 4-nitroquinoline-1-oxide (4NQO) chemical carcinogen for twenty weeks and then euthanized for microscopic evaluation of the tongue lesions. Increased pre-carcinogen norepinephrine concentrations and reduced basal corticosterone levels in the normal tissue microenvironment were predictive for oral squamous cell carcinoma (OSCC) occurrence. Likewise, increased pre-carcinogen norepinephrine levels in the normal microenvironment were associated a lower expression of pCDKN2a-p16 in OSCCs. Post-carcinogen levels of corticosterone and BDNF in oral leukoplakia tissues (precursor lesion of OSCC) and post-carcinogen corticosterone concentrations in OSCCs were higher than basal levels in the normal mucosa. Increased norepinephrine concentrations in OSCCs were associated to a greater tumor volume and thickness. Furthermore, higher levels of norepinephrine, ACTH and BDNF in OSCCs were associated to a lesser intensity of the lymphoplasmocytic infiltrate. This study shows that pre-carcinogen stress hormones levels in the normal microenvironment may be predictive for chemically induced cancer in rats. Moreover, chemical carcinogenesis can promote stressor-like effects with hormonal changes in the tissue microenvironment, which may be associated to tumor progression.

1. Introduction

Studies have shown that hormones derived from chronic stress can influence cancer progression (Antoni et al., 2006; Reiche et al., 2004). The two main pathways that have been investigated for mediating the effects of emotional stress on cancer are the hypothalamic-pituitary-adrenal (HPA) axis and the sympathetic nervous system (SNS) (Antoni et al., 2006; Reiche et al., 2004). HPA axis activation occurs when neurons of the paraventricular nucleus secrete corticotropin-releasing factor (CRF); in sequence, CRF stimulates adrenocorticotropic hormone

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(ACTH) secretion from anterior pituitary gland, which induces cortisol release from adrenal gland (Antoni et al., 2006). In parallel, SNS activation can induce a deregulated production of the catecholamines norepinephrine and epinephrine by the central nervous system (CNS) and adrenal gland (Antoni et al., 2006).

Stress hormones increase the tumor production of pro-inflammatory cytokines, chemokines and growth factors affecting cancer progression (Antoni et al., 2006; Reiche et al., 2004). Stress levels of norepinephrine and epinephrine, per example, enhance the gene expression of vascular endothelial growth factor (VEGF) (Lutgendorf et al., 2003; Thaker et al., 2006) and interleukin-6 (IL-6) (Bernabé et al., 2011; Lutgendorf et al., 2008b; Nilsson et al., 2007), molecules that increase angiogenesis and cancer cell proliferation, respectively. Likewise, these hormones deregulate the production of matrix metalloproteinases (MMPs) types 2 and 9 (Lutgendorf et al., 2008a; Sood et al., 2006; Yang et al., 2006), proteins that enhance tumor invasion and metastasis occurrence. SNS-induced catecholamine release suppresses the responses of cytotoxic T lymphocytes and natural killer (NK) cells (Inbar et al., 2011). Experimental studies have demonstrated that stress hormones can affect ovarian (Lutgendorf et al., 2003; Lutgendorf et al., 2008a; Nilsson et al., 2007; Sood et al., 2006; Thaker et al., 2006), breast (Ben-Eliyahu et al., 1991), lung (Melamed et al., 2005) and colon (Lointier et al., 1992) cancer progression. Clinical investigations have shown that patients with advanced-stage ovarian (Lutgendorf et al., 2008b), colorectal (Rich et al., 2005) and head and neck (Bernabé et al., 2012) cancer have increased cortisol concentrations compared to patients with early-stage disease or healthy controls. Few studies have demonstrated that stress hormones can act on regular cells promoting cancer development (Feng et al., 2012; Flint et al., 2013). In a recent study, chronic stress promoted tumor development (lymphomas and sarcomas) in male mice by the attenuation of p53 protein levels and transcriptional activity, which were mediated by increased corticosterone levels in the serum (Feng et al., 2012).

Brain-derived neurotrophic factor (BDNF) is a neurotrophin widely expressed in the CNS, where it acts as trophic factor for dopaminergic and cholinergic neurons. Evidence have shown that BDNF expression in the CNS can be modulated by stress (Lewin and Barde, 1996). BDNF and its receptor TrkB have an important role as autocrine modulators being expressed in several tissues (Lewin and Barde, 1996). Acute or chronic stress may increase plasma BDNF levels in preclinical models (Saruta et al., 2010; Tsukinoki and Saruta, 2012). BDNF expression is associated with tumor cell proliferation and invasion (Kupferman et al., 2010; Yang et al., 2005) and has been identified in head and neck (Kupferman et al., 2010), liver (Yang et al., 2005), prostate (Bronzetti et al., 2008) and bladder (Lai et al., 2010) cancer, suggesting a role of this neurotrophin in cancer pathogenesis.

Tumor microenvironment is an aberrant tissue environment composed of tumor epithelial cells and mesenchymal cells surrounded by extracellular matrix (ECM) (Hu and Polyak, 2008). Most solid tumors are innervated by sympathetic fibers, which can release norepinephrine in the microenvironment (Saloman et al., 2016). Although evidence show that stress and its neurohormones may influence cancer progression, there are no studies that have assessed stress hormones concentrations in the tissue microenvironment before cancer occurrence and its predictive value for carcinogenesis and tumor progression. Exposure to chemical or physical carcinogens may affect important cellular metabolic functions such as the production of stress hormones (Al-Wadei et al., 2012; Anna et al., 2007). However, it is still unclear how carcinogens could modulate stress hormones levels in the tumor microenvironment.

In the present study, we used a preclinical oral carcinogenesis model to test the hypothesis that physiological levels of stress hormones in the normal tissue microenvironment (before tumor induction) would have predictive value for chemically induced cancer occurrence. Furthermore, clinicopathological features and tumor progression-related genes were analyzed after tumor induction.

2. Materials and methods

2.1. Animals and maintenance conditions

All procedures with animals followed the NIH Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Welfare Committee at the Araçatuba Dental School (São Paulo State University - Unesp, Araçatuba, Brazil). Forty-eight male Wistar rats, with 12 weeks old, weighing between 250 and 300 g, were used for the experiments. The animals were group-housed (3 per cage) (25.9 × 47.6 × 20.9 cm, polypropylene), under constant room temperature (RT) (25 ± 2 °C), relative humidity (55 ± 3 °C), lighting (12-h light-dark cycle) and received food *ad libitum* (Purina, Paulínia, SP, Brazil) and drinking water.

2.2. Experimental design

In order to assess whether stress hormones levels in the tongue microenvironment would have predictive value for chemically induced oral carcinogenesis, we used a new methodology. The experiments were divided in three steps. In phase 1, all animals were submitted to a tissue collection for characterizing the stress hormones concentrations in the tongue microenvironment (pre-carcinogen hormonal levels). In phase 2, these same animals were underwent to chemically induced oral carcinogenesis. In phase 3, after 20 weeks of oral carcinogenesis, all animals were euthanized for evaluation of oral cancer occurrence and stress hormones concentrations in the microenvironment (post-carcinogen hormonal levels) (Fig. 1A).

2.3. Individual characterization of the stress hormones levels in the tongue microenvironment pre-carcinogenic induction

2.3.1. Anesthetic induction

In the first phase of research, to characterize stress hormones levels in the tissue microenvironment all animals were submitted to a tongue biopsy under inhalatory anesthesia. Anesthetic induction was performed in an induction-chamber with 5% isoflurane in 100% oxygen (3L/min flow) connected to an open, non-rebreathing, circuit (Anesthesia equipment Nikkei K - Takaoka, São Paulo, SP, Brazil). After loss of muscle tone (ventral decubitus position), the animal was removed from the induction-chamber and maintained under anesthesia with an adapted face mask connected to the same induction system. Anesthetic maintenance was performed with 3% isoflurane and oxygen flow of 2L/min during the surgical procedure.

2.3.2. Tongue biopsy pre-carcinogenic induction

With the animal anesthetized, the tongue was pulled with a suture thread (Fig. 1B) and a biopsy was performed in the posterior region of the tongue dorsum with a 4 mm circular punch (Fig. 1C) allowing the removal of a lingual mucosa fragment (Fig. 1D). This area was chosen to measure the stress hormones concentrations, since it is the main location where the carcinomas develop in the chemical carcinogenesis model used in this study. After removing the tongue fragment, the surgical wound was sutured with 4.0 silk thread (Ethicon Johnson & Johnson, São José dos Campos, SP, Brazil). The tissue sample was rinsed with physiological solution and stored at −80 °C for hormones measurements. The tissue collection was always performed in the morning period between 8 am and 12 pm. During the three weeks postoperative period following tongue biopsy, the animals were fed with water-softened chow.

2.3.3. Measurements of the stress hormones concentrations in the tongue microenvironment

2.3.3.1. Sample preparation. For measuring stress hormones levels in the tongue microenvironment, the tissue samples were homogenized in a PBS buffer with a protease inhibitor cocktail (Calbiochem, San Diego,