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Antiangiogenic and finasteride therapies: Responses of the prostate microenvironment in elderly mice

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ABSTRACT

Aims: The aim of this study was to evaluate the structural and molecular effects of antiangiogenic therapies and finasteride on the ventral prostate of senile mice.
Main methods: 90 male FVB mice were divided into: Young (18 weeks old) and senile (52 weeks old) groups; finasteride group: finasteride (20 mg/kg); SU5416 group: SU5416 (6 mg/kg); TNP-470 group: TNP-470 (15 mg/kg,) and SU5416 + TNP-470 group: similar to the SU5416 and TNP-470 groups. After 21 days, prostate ventral lobes were collected for morphological, immunohistochemical and Western blotting analyses.
Key findings: The results demonstrated atrophy, occasional proliferative lesions and inflammatory cells in the prostate during senescence, which were interrupted and/or blocked by treatment with antiangiogenic drugs and finasteride. Decreased AR and endostatin reactivities, and an increase for ER-α, ER-β and VEGF, were seen in the senile group. Decreased VEGF and ER-α reactivities and increased ER-β reactivity were verified in the finasteride, SU5416 groups and especially in SU5416 + TNP-470 group. The TNP-470 group showed reduced AR and ER-β protein levels.
Significance: The senescence favored the occurrence of structural and/or molecular alterations suggesting the onset of malignant lesions, due to the imbalance in the signaling between the epithelium and stroma. The SU5416 + TNP-470 treatment was more effective in maintaining the structural, hormonal and angiogenic factor balance in the prostate during senescence, highlighting the signaling of antiproliferation *via* ER-β.

Introduction

Senescence is the period of life that is associated with significant changes in the hormonal environment of the different animal species, and is a causative factor of morphological and functional changes in the prostate (Morales, 2002; Roy-Burman et al., 2004). According to Lau et al. (2003), the changes found in the prostate of senile mice are similar to those observed in benign prostatic hyperplasia in humans. Zhao et al. (2002) associated senescence to differences in gene expression and in the immunolocalization of growth factors in the prostate of mice, relating the growth factors such as TGF and EGF in the pathogenesis of age-related diseases, such as cancer and benign hyperplasia.

It is known that there is a progressive decline in the testosterone and dehydroepiandrosterone (DHEA) levels, which is followed by an increase of estrogen resulting in a hormonal imbalance in elderly men (Morales, 2002). Testosterone (T) and dihydrotestosterone (DHT) are the main androgens, which induce prostate differentiation (Hsing, 2001; Toorians et al., 2003). DHT is a result of the conversion of testosterone by the 5α-reductase enzyme and these hormones could be involved in different prostatic functions (Toorians et al., 2003). Although the prostate is primarily regulated by androgens, its development is sensitive to estrogens, which act synergistically to testosterone, influencing both the normal functions of the body as well as the deleterious alterations (Weihua et al., 2001; Cunha et al., 2002). The estrogen biosynthesis is originated from androgenic substrate by means of aromatase action (O'Donnell et al., 2001). The estrogenic action occurs *via* receptors, and two of these isoforms (ER-α and ER-β) are present in different male reproductive tissues, including the prostate (Pelletier and El-Alfy, 2000). Thus, taking into consideration the androgenic role in the prostatic development and occurrence of lesions associated to hormone imbalance, finasteride has been used as one of the 5α-reductase II inhibitors in the treatment of symptoms in the lower urinary tract related to benign prostatic hyperplasia in human beings (Thompson et al., 2009). Through the suppression of DHT, finasteride reduces the growth of prostatic tissue by reducing glandular and fibromuscular tissue and

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plays a role in the angiogenic process by decreasing the VEGF expression and microvessel density in suburethral prostatic tissue (Häggström et al., 2001; Pareek et al., 2003).

Another important topic for prostate lesions is related to the angiogenesis blockage which has been identified as a new anticancer strategy, considering that neovascularization is required to sustain the growth of solid tumors (Aragon-Ching et al., 2010). According to Folkman et al. (1998) angiogenesis inhibitors are divided into two classes: direct and indirect inhibitors. The direct angiogenesis inhibitors act in the vascular endothelial cells by preventing them from responding to several pro-angiogenic stimuli. Meanwhile, the indirect inhibitors of angiogenesis interfere in the pro-angiogenic communication between the tumor cells and the endothelial cells (Folkman et al., 1998).

SU5416 is a tyrosine kinase lipophilic synthetic inhibitor (TKI), indirect inhibitor of angiogenesis that blocks the phosphorylation of VEGF receptor, and has potent antiangiogenic properties in preclinical studies (Fong et al., 1999; Abdollahi et al., 2003; Christensen, 2007). On the other hand, the TNP-470 direct angiogenesis inhibitor, a fumagillin analog, has a potent cytostatic action in endothelial cells and also significant antitumor properties (Masiero et al., 1997; Folkman, 2005).

The complexity of the angiogenic process in malignant diseases suggests that the combination of direct and indirect antiangiogenic agents can be an effective way of treating and or preventing lesions as well as the use of finasteride. Thus, the aim of this study was to evaluate the effects of antiangiogenic and finasteride therapies during the senescence on the molecular and structural biology of the mouse prostate.

Materials and methods

Animals and experimental procedure

Ninety male FVB mice were used and divided into six experimental groups. The young (18 weeks old) and the senile (52 weeks old) control groups: received subcutaneous doses of 5 mL/kg 0.9% saline solution; the finasteride group: received subcutaneous doses of 20 mg/kg finasteride (F1293 — Sigma-Aldrich) modified from Tutrone et al. (1993) and Gonzales et al. (2008); the SU5416 group: received intraperitoneal doses of 6 mg/kg SU5416 (Cat.3037 — Tocris Bioscience) modified from Strieth et al. (2006); the TNP-470 group: received subcutaneous doses of 15 mg/kg TNP-470 (Cat.3750 —Tocris Bioscience,) modified from Matsusaka et al. (2000); and the SU5416 + TNP-470 group: received the same treatment as the SU5416 and TNP-470 groups. All drugs were diluted in dimethylsulfoxide (DMSO). The drugs were administered every other day during 21 days. The animals were anesthetized with 2% xylazine hydrochloride (5 mg/kg i.m.; König, SãoPaulo, Brazil) and 10% ketamine hydrochloride (60 mg/kg, i.m., Fort Dodge, Iowa, USA) and sacrificed. All the animals received water and the same solid diet *ad libitum*. This study was approved by the Ethics Committee on Animal Use (CEUA) — UNICAMP, protocol 2391–1.

Morphological analysis

Prostate ventral lobe samples taken from five animals from each group were collected and fixed in Bouin's solution for 24 h. The animals were the same used to immunohistochemistry, determination of proliferative index and density microvessel. Then, tissues were rinsed in 70% ethanol and dehydrated. The materials were embedded in plastic polymers (Paraplast Plus, ST. Louis, MO, USA) and stained with hematoxylin–eosin, Masson Trichrome, and Toluidine Blue and photographed in a Nikon EclipseE-400 light microscope (Nikon, Tokyo, Japan) and morphological aspects were evaluated.

The inflammatory infiltrates were identified in the stromal, periglandular and glandular areas according to specific locations and categorized considering the relation between percentage of inflammatory infiltrate foci × total measured glandular area. The categories were focal (<10%), multifocal (10–50%) and diffuse (>50%) (modified from Nickel et al., 2001). The analyses were measured in a 40× objective lens in 10 fields from 5 animals in each experimental group.

Immunohistochemistry

Prostate ventral lobe samples were collected from five animals in each experimental group. The animals were the same used for light microscopy, determination of proliferative index and determination of density microvessel. Samples were evaluated using immunohistochemistry for the detection of the androgen receptor (AR- rabbit polyclonal IgG; sc-816, Santa Cruz Biotechnology); estrogen alpha (ER-α- mouse monoclonal IgG; 1D5, Dako Cytomatation) and beta receptors (ER-β- rabbit polyclonal IgG; 06-629, Upstate), vascular endothelial growth factor (VEGF- rabbit polyclonal IgG; sc-53462, Santa Cruz Biotechnology); endostatin (mouse monoclonal IgG; ab64569, Abcam), Ki67 (polyclonal rabbit IgG, ab9260, Abcam) and CD31 (rabbit polyclonal IgG; sc1506-r, Santa Cruz Biotechnology), the pattern protocols were similar to those described by Hetzl et al. (2013) and Montico et al. (2011) and the primary antibodies were diluted in concentration from 1:50 to 1:100. The sections were incubated with secondary conjugate HRP antibody from the Envision kit (Dako) for 40 min and subsequently developed with 3,3′-diaminobenzidine (DAB). Harris' hematoxylin was used for counter-staining. The frequency of antigen immunoreactivity was graded as 0 for negative staining (0%), 1 for weak staining (<33%), 2 for moderate staining (33–66%), and 3 for intense staining (>66%) according to the frequency and positivity of antigens in sectioned tissues (modified from Tomas and Kruslin, 2004; Tuxhorn et al., 2002a,b). The immunohistochemical analyses were followed by a negative control parameter in which the primary antibody was not used.

Cell count positive Ki-67 and determination of proliferative index

Proliferative index was obtained by counting the Ki-67 positive cells. The prostate ventral lobe samples of five animals from each experimental were used. The same animals were used for light microscopy, immunohistochemistry, determination of proliferative index and determination of density microvessel. Next, ten fields for each specimen were evaluated with a 100× objective lens and the proliferative index was determined by dividing the number of the Ki-67 positive cells by the total number of cells found in the microscopic field. The number of nuclei counted was from 750 to 1000 in the different experimental groups.

Detection of apoptosis and determination of the apoptotic index

Prostate ventral lobe samples of five animals from each experimental group were collected and fixed in 4% paraformaldehyde. After, the samples were included in plastic polymers (Paraplast Plus TS. Louis, MO, USA), sectioned with 5 μm thickness and submitted to DNA fragmentation reaction detection. The DNA fragmentation was detected using the detection system for fluorescent apoptosis (Promega, Madison, WI, USA) and Feulgen. Apoptotic nuclei were identified and photographed using the Olympus IX71 inverted-II microscope equipped with fluorescence (Olympus, California, USA).

The sections were submitted to hydrolysis with 4NHCl for the Feulgen reaction, and treated with Schiff reagent. After washing with water, the sections were dehydrated and mounted on slides. Next, ten fields of each animal were analyzed with a 100× objective lens and the apoptotic index was determined by dividing the number of apoptotic nuclei by the total number of nuclei found in the microscopic fields. The number of nuclei counted was from 750 to 1000 in the different experimental groups. Apoptotic nuclei were identified by characteristics such as pyknosis and or nucleolar fragmentation.