Nitric oxide synthase: a study of its expression as a molecular marker in relation to prostate cancer


ABSTRACT

Background: A descriptive, comparative, prospective study was carried out to observe neuronal nitric oxide synthase (nNOS) expression in prostate tissue presenting with cancer and hyperplasia.

Methods: Tissue samples were taken from patients diagnosed with benign prostatic hyperplasia and prostate cancer within the time frame of 2005 to 2009. The samples were stored in the Pathology Department of the Hospital Central Militar, according to department inclusion, exclusion and elimination criteria.

Results and Conclusions: Relative density was obtained through immunohistochemistry and a reduction of nNOS was observed in tissues with prostate cancer when compared with its expression in tissues with benign prostatic hyperplasia (BPH).

Keywords: Prostate cancer, molecular expression, markers, nitric oxide synthase, Mexico.

RESUMEN

Introducción: Se realizó un estudio prospectivo, comparativo, descriptivo, para observar la expresión de sintasa de óxido nítrico neuronal (nNOS), en tejido prostático con cáncer e hiperplasia.

Métodos: Se tomaron las muestras de tejidos correspondientes a los pacientes diagnosticados dentro del periodo comprendido entre 2005 y 2009, almacenados en el Departamento de Patología del Hospital Central Militar, de acuerdo con los criterios de inclusión, exclusión y eliminación del mismo.

Resultados y Conclusiones: Por medio de la técnica de inmunohistoquímica se obtuvo la densidad relativa, donde se observó una disminución de nNOS en tejidos con cáncer de próstata (CaP) en comparación con la expresión en los tejidos de hiperplasia benigna prostática (BHP).

Palabras clave: Cáncer de próstata, expresión molecular, marcadores, sintasa de óxido nítrico, México.
INTRODUCTION

In Mexico, prostate cancer (CaP) is in second place after skin cancer with respect to cancer incidence, and holds second place after cervical cancer in relation to mortality from cancer. After the age of 50 years, prostate cancer incidence increases 3 or 4 times every 10 years. The molecular mechanisms that are related with CaP development and progression have not been studied in their entirety. As stated in the literature, there is a close relation between oxidative stress and the development of cancer.1,2

Benign prostatic hyperplasia (BPH) etiology is multifactorial. Two factors are presently recognized: age and the presence of androgens. Nevertheless, it is believed that other factors may be involved in this process.3 The most recent theories support the idea of a hormonal imbalance of estrogens/androgens or of the existence of prostatic growth factors with a permissive role of the hormonal environment.4

The development of BPH in men is considered to be a normal aspect of aging and is hormonally dependent on testosterone and dihydrotestosterone (DHT) production.5

An increase in 5-α-reductase has been demonstrated in the periurethral zone and in stroma, two areas that are fundamental in hyperplasia development, and there is also evidence of reduced DHT catabolism.6

In addition, it is argued that estrogen participates in the origin and maintenance of prostatism, supported by the following data: 1) estrogen receptors are more elevated in stroma than in epithelium, 2) there has been experimental demonstration of the synergic effect of estrogens and androgens in hyperplasia production in the dog, and 3) stimulus with 17-estradiol increases the number of androgen receptors in hyperplastic tissue.7

A variety of growth-promoting factors have been isolated from prostate tissue and they include: epidermal growth factor (EGF), fibroblast growth factor (FGF), platelet-derived growth factor (PDGF), transforming growth factors (TGF-alpha, TGF-beta), and osteoblast growth factors. The factors that are most dependent on androgen action are: EGF and TGF-alpha. Lepor et al. showed that concentrations of these factors are greater in hyperplastic tissue.8-15

The specific causes determining the appearance of CaP are presently unknown, but an association with genetic and environmental factors has been observed.15,16

Prostate cancer (CaP) onset and progression are influenced by androgens. A diet rich in fats promotes carcinogenic cell growth in the prostate and fats are a source of free radicals that favor the appearance of CaP.17-19

In a study in the American Journal of Epidemiology in 2004, associations with advanced cancer were more homogeneous, suggesting a relation to saturated fats, but not to linoleic acids, polyunsaturated fats, alpha-linoleic acids, eicosapentaenoic acids, and docosahexaenoic acids.18

There are genetic polymorphisms associated with CaP risk, such as the 5-α-reductase type 2 and the 3-hydroxysteroid-dehydrogenase type 2 genes. The androgen receptor gene presents two polymorphisms that play a role in CaP onset and progression.20-23

There is a factor located in the long arm of chromosome 1 (1q24-25) that increases the risk for developing this cancer at an earlier age.14,15 The isoflavonoid genistein (that inhibits 5-reductase), leafy green vegetables that contain isothiocyanate sulforaphane, retinoids such as the lycopennes, and the cholesterol biosynthesis inhibitors are among the protective factors. Risk could also be reduced by alpha-tocopherol, which is an antioxidant and cell membrane protector (vitamin E), selenium, and vitamin D, which is a steroid hormone that inhibits CaP cell line proliferation and induces differentiation.24-33

METHODS

Tissues were used that came from patients diagnosed with benign prostatic hyperplasia (BPH) and prostate cancer (CaP) that underwent surgery in the Urology Operating Room of the (within the period of September 2005 to April 2009). The samples that fit the inclusion, exclusion, and elimination criteria were later requested from the head of the hospital’s Department of Pathology. The clinical case records of the patients were reviewed and specific data were transferred to a data collection sheet.

Inclusion criteria were: tissue from patients with histopathologic BPH and CaP diagnoses obtained from the Pathology storage room and registered between the years of 2005 and 2009.

Elimination criteria were: Tissue that was not adequately conserved during its transport or storage, as well as insufficient sample.

The immunohistochemical determination of nNOS expression was carried out in tissues presenting with CaP and BPH and their expression in both groups was compared.

Immunohistochemistry: Using a light microscope CaP and BPH tissue samples were fixed by formalin immersion (pH=7.4) and were embedded in paraffin. For the histologic analysis, tissue sections (3 μm) were stained with hematoxylin and eosin (H&E). The tissue sections were then stained with periodic acid-Schiff (PAS)
to show polysaccharides, mucopolysaccharides, and glycoproteins of the cell membrane.

The slices were incubated with Schiff’s reagent for 5 minutes and counterstained with hematoxylin for 30 seconds. The histologic profile of 5 randomly selected fields was registered using KS-300 software (Carl Zeiss, Jena, Germany). The percentage of damaged area with histopathologic alterations was obtained (magnification x400). For the immunohistochemistry, the tissue sections (3 μm) were deparaffinized and heated to unmask the antigen sites; peroxidase endogenous activity was blocked with 0.03% of H₂O₂ in absolute methanol. Tissue sections were incubated all night long at 4ºC at a 1:100 dilution of anti-CYP4F11 and at a 1:50 dilution for anti-CYP8A1 in TRIS solution. The primary antibody was removed and washed repeatedly with TRIS. The slices were incubated with a 1:500 dilution of polyclonal rabbit antibody as the secondary antibody and washed repeatedly with TRIS.

The bound antibodies were detected with the avidin-biotin complex (Vectastain® ABC kit) and diaminobenzidene as substrate. After repeated washes with TRIS, the slices were counterstained with hematoxylin. All slices were incubated under the same conditions, at the same antibody concentration, and in the same run, making the immunostaining comparable. All specimens were examined using an Axiovert 200M light microscope (Carl Zeiss, Jena, Germany). For the automated morphometric analysis, the percentage of positive cells (brown) was determined with a KS-300 3.0 computerized image analyzer (Carl Zeiss, Jena, Germany). This equipment automatically detects positive cells, calculating their percentage per field. Five random fields were studied at a magnification of x100 (total area 1,584,000 μ²). The results were expressed as percentage.

STATISTICAL ANALYSIS
The statistical significance of nNOS expression determined as relative density between the CaP and BPH groups was statistically processed using Prism 3.0 software with the Fisher “F” test and densitometric values were presented as arithmetic mean ± standard error of densitometric units. Differences were considered statistically significant when \( P < 0.05 \).

RESULTS
From a total of 84 patients, 40 patients had a histopathologic diagnosis of prostate cancer (CaP) and 44 had a histopathologic diagnosis of benign prostatic hyperplasia (BPH). In the group of patients diagnosed with CaP, the mean age was 65.3 years and the mean preoperative prostate specific antigen (PSA) concentration was 11.9 ng/mL; Gleason score was 2 in 1 case (2%), 3 in 1 case (2%), 4 in 7 cases (18%), 5 in 6 cases (15%), 6 in 6 cases (15%), 7 in 10 cases (25%), 8 in 6 cases (15%), and 9 in 3 cases (8%); radical prostatectomy was performed in the 40 patients (100%) due to the diagnosis of clinically localized cancer.

In the group of patients with the established diagnosis of BPH, the mean age was 67.5 years and the mean preoperative PSA concentration was 5.8 ng/mL. Transurethral resection of the prostate (TURP) was performed on all 44 patients and radical prostatectomy was carried out only in some of the cases. The nNOS immunoreactivity in glandular tissue with BPH expressed in relative densitometric units was 50160 ± 20000 compared with 28990 ± 9923 in tissue with CaP (<0.0001). The relative density in the stromal tissue with BPH was 38480 ± 16900 compared with tissue with CaP that showed 10530 ± 2126 (<0.0001) (Figure 1).

DISCUSSION
In our study we observed greater nNOS expression in glandular and stromal tissue with BPH, 50160 ± 20000 and 38480 ± 16900, respectively, compared with tissues with CaP, 10530 ± 2126 and 38480 ± 16900 (\( P < 0.0001 \)). This is consistent with the results obtained in studies reported in the literature in which nNOS expression in tissues with cancer was diminished.¹⁶-³⁰
Nitric oxide (NO), with its consequent production of free radicals, has been described to be associated with apoptosis inhibition. 3135 The production of NO is regulated by 3 isoenzymes: inducible nitric oxide synthase (iNOS), endothelial nitric oxide synthase (eNOS), and neuronal nitric oxide synthase (nNOS). The iNOS isoenzyme is mediated by transcription and largely contributes to the formation of this radical, with the possibility of creating pathologic concentrations, while the eNOS and nNOS isoenzymes produce lower physiologic concentrations.33-35

This concurs with the result of our study, given that if a greater concentration of free radicals resulting from the oxidative stress of NO is possible, nNOS should not be overexpressed because it contributes poorly to NO production and can even be reduced, due to the greater iNOS activity.36

Finally, another mechanism that could explain the reduction of nNOS expression is induction by cytokines in tumor processes that increases NO production, which activates the soluble guanylate cyclase enzyme, resulting in an increase of cyclic guanosine monophosphate (cGMP). This increase can lead to the stimulation of kinase, one of the cGMP-dependent proteins, the altered phosphorylation of many endogenous proteins, reduced phospholipase C enzyme activity, and cytosolic calcium reduction. Cytosolic calcium reduction causes the 40-50 amino acid loop insert that inhibits nNOS. 34-37

**CONCLUSIONS**

The area percentage marked per field of the nNOS enzyme is reduced in tissues with prostate cancer when compared with tissues with BPH. Our study is consistent with those reported in the literature; NO is related to induction factors in cancer, but in its synthesis, nNOS is a poor contributor to its formation.

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