Endothelial nitric oxide synthase expression determination in prostate cancer and benign prostatic hyperplasia tissue

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ABSTRACT

Background: Recent reports have placed prostate cancer in third place worldwide. In Mexico it is the principal cause of death by cancer after skin cancer. Oxidative stress has been observed to play a role in cancer etiology. The present study evaluates whether or not there is any alteration in endothelial nitric oxide synthase, an oxidative stress marker, in prostate cancer and benign prostatic hyperplasia.

Methods: Thirty-nine benign prostatic hyperplasia tissue samples and forty-two prostate cancer tissue samples were obtained and conditions were standardized in order to immunohistochemically detect the presence of endothelial nitric oxide synthase in the tissues.

Results: The percentage of area immunoreactive for endothelial nitric oxide synthase was 1.1779±0.25% in prostate cancer tissue and 3.162±0.32% in benign prostatic hyperplasia tissue.

Conclusions: Densitometric data for endothelial nitric oxide synthase immunoreactivity in prostate cancer glandular tissue were lower than those obtained in...
INTRODUCTION

In Mexico prostate cancer (CaP) is the second most common neoplasia in men after skin cancer and in 2001 it was in first place as cause of death from malignant tumor in men.1

Oxidative stress is considered to be a state in which there is an excess of reactive oxygen species (ROS)9 as well as a reduction of endogenous antioxidants.2,3 Lesions produced by free radicals are manifested in this state of oxidative stress. The free radicals react with lipids, proteins, carbohydrates and DNA of the cells, but also with components of the extracellular matrix. This reaction can result in irreparable damage and if it is very extensive can lead to cellular death. Oxidant and antioxidant imbalance is associated with the physiopathology of various diseases. The biological aging process is accelerated in direct relation to the magnitude of oxidative stress.4

Oxidative damage has recently been thought to play a critical role in various clinical conditions among which are malignant diseases. ROS can cause DNA oxidation and protein damage, tumor suppressor gene damage, and an increase in proto-oncogene expression. Cancer has a pro-oxidative change in the oxidoreduction state. ROS are potential carcinogens because they facilitate mutagenesis, in addition to tumor promotion and progression. Even normal cells show an increase in the proliferation and expression of genes related to growth if they are exposed to hydrogen peroxide or superoxide.

Nitric oxide synthase (NOS) is an enzyme that contains a group with a similar sequence to cytochrome P-450 reductase. In humans there are 3 NOS isoforms located in chromosome 17: neuronal nitric oxide synthase (nNOS) and endothelial nitric oxide synthase (eNOS), constitutive isoforms that are regulated by calcium concentrations of 70-100 nM, and inducible nitric oxide synthase (iNOS), an inducible isoform that only requires calcium concentrations of 30-70 nM because, unlike other isoforms, it is constitutively bonded to calmodulin. In addition to being induced by lipopolysaccharides (LPS) and cytokines such as INFγ, FNTα and IL1, iNOS is constitutively present in the bronchial epithelium, the kidney and certain fetal tissues. eNOS is constitutively regulated in endothelium through the tension exerted by blood flow on the vascular wall (friction tension) but can be induced through chronic exercise or pregnancy.

NOS expression regulation is controlled at both the transcriptional as well as the post-transcriptional levels. These enzymes are phosphorylated by a variety of protein kinases and by tetrahydrobiopterin (H4B).

The functional role of nitric oxide (NO) can vary depending on each cellular strain and enzyme isotope that produces it. The main function of eNOS is vascular regulation. The NOS inducible isoform is detected mainly in immune cells and is activated mainly by the cell wall component of bacteria.5

iNOS can be found in different tissues undergoing inflammatory processes. The resulting NO creation can be beneficial, but at times can be involved in pathological processes, partly depending on the amount of NO that is generated and on the prevalence of oxidizing conditions.

de próstata fueron menores que los obtenidos en tejido similar de hiperplasia prostática benigna. De acuerdo con lo anterior, la determinación de eNOS podría ser utilizada como un marcador de diferenciación de etapas tempranas del cáncer de próstata.

Palabras clave: cáncer de próstata, hiperplasia prostática benigna, sintasa de óxido nítrico endotelial, México.

Abbreviationa

CaP: Prostate cancer
ROS: Reactive Oxygen Species
ERNs: Reactive Nitric Species
HPB: Benign Prostatic Hyperplasia
eNOS: Endothelial Nitric Oxide Synthase
in the tissue. Cytokine immune stimulation or bacterial pathogen activity activates iNOS and generates high levels of NO through the activation of inducible nuclear factors including NFK-B. NO overproduction in live organisms is determined at different cellular levels. Treatments of this NO overproduction are sometimes achieved without affecting its physiological levels.

It is worth mentioning that NOS is involved in the promotion of tumor growth. Therefore there are important differences in eNOS and iNOS expression regulation in CaP tissue, compared with benign prostatic hyperplasia (BPH) tissue in such a way that this regulation can be used in diagnosing the disease.

## METHODS

### Study subjects

Samples were collected from September 2008 to April 2009 from patients treated at the Hospital Central Militar that met the following inclusion criteria: a) patients diagnosed with CaP that were radical prostate surgery candidates, b) patients diagnosed with CaP that had prostatic tunnelization indication, and c) patients diagnosed with BPH that had transurethral resection of the prostate (TURP) indication. Exclusion criteria: a) patients that did not give their authorization to participate in the procedure and b) patients that were not TURP candidates. Elimination criteria: a) patients whose concentration and purity of extracted and purified RNA was not adequate for the study and b) tissue that suffered degradation of nucleic material during transport.

The quantity of tissue necessary for the study was 1-5 g. Samples were obtained immediately after surgical intervention. Transportation time was a maximum of one hour from the time sample was taken to its delivery to the Biología Molecular de la Escuela Médico Militar laboratory. Samples were kept there at a temperature of -70°C in the Revco® (Legaci ULT2186 3-35 Dupont SVVA Refrigerants) ultrafreezer.

### Immunohistochemistry

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

Slices were incubated with periodic acid for 5 min and washed with distilled water. Slices were incubated with Schiff’s reactive for 5 minutes and counterstained with hematoxylin for 30 seconds. The histological profile of 5 randomly selected fields was registered using KS-300 software (Carl Zeiss, Jena, Germany). The percentage of damaged area with histopathological changes was obtained (400x magnification). For immunohistochemistry tissue sections (3 mm) were deparaffinized and warmed to unmask antigenic sites. Endogenous peroxidase activity was blocked with 0.03% of H2O2 in absolute methanol. Tissue sections were incubated overnight at 4°C at a dilution of 1:200 of monoclonal antibody against eNOS in TRIS solution. Primary antibody was removed and 2 washes with TRIS were done, one after the other. Slices were incubated in a dilution of 1:500 rabbit polyclonal antibody as secondary antibody and wash was repeated twice with TRIS. Bonded antibodies were detected with avidin-biotin complex (Vectastain ABC-kit) and diaminobenzidine as substrate. After repeated washes with TRIS slices were counterstained with hematoxylin. All slices were incubated under the same conditions with the same antibody concentration in the same run, so immunostain was comparable. All specimens were examined under Axiovert 200M light microscope (Carl Zeiss, Jena, Germany). For automatized morphometric analysis the percentage of positive cells (brown colored) was determined with a KS-300 3.0 computerized image analyzer (Carl Zeiss, Jena, Germany). This equipment automatically detects positive cells, determining their percentage per field. Five random fields were studied at a magnification of 100x (1,584,000 m2 total area). The results were expressed as percentages.

### Data analysis

eNOS immunoreactivity data was analyzed with Student t test and data were expressed as mean ± standard error (SEM). The CaP and BPH groups were compared and statistically significant difference was considered to exist when P <0.05. Statistical tests were applied using the Graph Prism version 3.32 program. Data were expressed as mean ± standard deviation.

## RESULTS

Biological samples were collected from 81 patients. Forty-two (51.85 %) of those patients presented with established CaP diagnosis (Gleason score 4.47 ± 3.1, age 67.77 ± 8.65 years, PSA = 35.29) and 39 (48.14%) patients presented with established BPH diagnosis (age 64.37 ± 11.07 years, PSA = 26.32).

### Quantitative eNOS expression in BPH and CaP in stromal tissue:

## DISCUSSION

Evidence shows that changes in reactive oxygen species (ROS) and reactive nitrogen species (RNS) promote neoplastic growth since these reactive species are involved in signal transduction, and carry out important activities in biological function regulation. Therefore it has been
suggested that nitric oxide (NO) and protein nitration can have important effects on tumor development.

On the other hand, in various pathological entities such as cancer, expression of the three NO synthase isoforms (inducible, iNOS; endothelial, eNOS; and neuronal, nNOS) has been found to be elevated as mentioned by Klotz T. Blochw et al. In 1998 they suggested that NO increases due to iNOS, having an important function in the development of tumors and of angiogenesis. These synthases have not been sufficiently studied in CaP. There is indication that the concentrations of these enzymes are low in benign prostate tissue and those authors conclude that epithelial iNOS expression can be used as a specific CaP marker since to a certain extent NO has an important function in the development of the disease. In 1999 Gradini R. et al partly lent support to this conclusion because they found that there was an increase in iNOS expression in BPH tissue - something that is not observed in normal prostate tissue.

In 2007 Cronauer et al showed that chronic inflammation increases the risk of developing certain types of cancer, including CaP. They found that iNOS is implicated in the development and increase of the inflammatory process since NO production is increased due to this enzyme. This influences the presence of acute or chronic inflammation including the presence of autoimmune diseases. In the case of tumor genesis, as in the present study, it could be influencing an increase in acute as well as in chronic inflammatory processes that, as mentioned above, can be implicated in neoplastic etiopathogeny. In the literature, however, the role of iNOS activity in the majority of diseases is not well understood and there is still no consistent data found in the different studies.

In the same study by Cronauer et al, they found that in CaP biopsy and immunohistochemistry iNOS protein expression in tumor cells is strongly related to 3-NT concentration, indicating that it plays a very important role in tumor process, development, and progression. They suggest that iNOS intratumoral activity favors cell development and that these cells are capable of proliferating independently, in this way promoting the development of neoplastic cells. These observations...
seem to point to the importance of NO in signaling oncogenesis.

■ CONCLUSIONS

The above results appear to suggest that eNOS plays an important role in CaP development.

BIBLIOGRAPHY