Regulation of antioxidant enzymes as prostate tumor markers

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ABSTRACT

Background: Prostate cancer has recently been reported to be in third place worldwide; in Mexico it is the principal cause of death by cancer in men after lung cancer. Oxidative stress has been observed to play a role in cancer etiology. Catalase is a peroxisome-specific antioxidant enzyme in mammals and is an important inflammation and oxidative stress regulator. Superoxide dismutase (SOD1) is an antioxidant enzyme that facilitates dismutation of oxygen radicals to hydrogen peroxide and also catalyzes pro-oxidant reactions. In the present study, catalase and SOD-1 antioxidant enzyme expression was determined in prostate cancer and benign prostatic hyperplasia.

Methods: A total of 40 samples of benign prostatic hyperplasia tissue and 40 samples of prostate cancer tissue were obtained. Conditions were standardized to immunohistochemically detect the presence of catalase and SOD-1 in the tissues.

Results: The percentage of the area that was immunoreactive to catalase and to SOD-1 was greater in prostate cancer tissue than in benign prostatic hyperplasia tissue.

Conclusions: Densitometric expression and percentage of the area marked with catalase and SOD-1 antioxidant enzymes was determined.

RESUMEN

Antecedentes: Informes recientes ubican al cáncer de próstata en el tercer lugar mundial; en México, es la principal causa de muerte por cáncer en los hombres después del cáncer de piel. En la etiología del cáncer se ha observado el papel del estrés oxidativo en el desarrollo del mismo. La catalasa es una enzima antioxidante específica de los peroxisomas de los mamíferos y es un importante regulador del estrés oxidativo y de la inflamación. La superóxido dismutasa (SOD1) es una enzima antioxidante que facilita la dismutación de radicales de oxígeno a peróxido de hidrógeno, y también cataliza las reacciones pro-oxidantes. En el presente trabajo, determinamos la expresión de enzimas antioxidantes catalasa y SOD-1 en cáncer de próstata e hiperplasia prostática benigna.

Métodos: Se obtuvieron 40 muestras de tejido prostático benigno y 40 de cáncer de próstata. Se estandarizaron las condiciones para detectar por inmunohistoquímica la presencia de catalasa y SOD-1 en los tejidos.

Resultados: El porcentaje de área inmunorreactiva a catalasa y a SOD1 fue mayor en cáncer de próstata que en hiperplasia prostática benigna.

Conclusiones: La expresión densitométrica y de porcentaje de área marcada por campo de enzimas antioxidantes...
enzymes per field was higher in prostate cancer tissue than in benign prostatic cancer tissue.

Key words: prostate cancer, benign prostatic hyperplasia, catalase, SOD1, Mexico.

INTRODUCTION

Presently in Mexico, prostate cancer (CaP) is in second place in regard to frequency of neoplasia in men following skin cancer. It is also the second cause of death by cancer in men after lung cancer. In the United States, more than 200,000 men are diagnosed with CaP annually and nearly 300,000 die from this disease each year. In the United States in 2006, there were 234,460 new cases of CaP and 27,350 men died from this disease.

The high incidence and prevalence of CaP is due to multiple factors. These risk factors include age, family history and race. Exposure to environmental factors also plays an important role. Although the exact environmental factors are not clear, diet (especially those rich in animal fats such as red meat and diets low in antioxidants such as selenium and vitamin E), work involving industrial chemicals, sexually transmitted infections and chronic prostatitis have been implicated in different degrees.

CaP incidence is higher in African Americans, followed by white Americans. East Asians living in their countries have a much lower incidence compared with those residing in the United States. However East Asians who live in the United States have a lower CaP incidence when compared with Caucasians and black people.

CaP detection has improved greatly with the introduction of prostate specific antigen (PSA), transrectal ultrasound of the prostate and biopsy techniques. There is an 80-90% relation of CaP with genetic and environmental factors but the understanding of their association with CaP is still deficient.

Recently the induction of oxidative stress by means of reactive oxygen species (ROS) and reactive nitrogen species (RNS) that arises during inflammation has been linked to CaP. These findings suggest that antioxidants regulate this stress and probably play a significant role in CaP prevention. Catalase is an enzyme that is present principally in peroxisomes in mammalian cells. It is a tetrameric enzyme comprised of four 60-kDa subunits that contain a heme group and nicotinamide adenine dinucleotide phosphate (NADPH) in their active center. Catalase performs two enzymatic activities that depend on H$_2$O$_2$ concentration. If H$_2$O$_2$ concentration is high, catalase acts as a catalyst, breaking down H$_2$O$_2$ and producing H$_2$O and O$_2$ (catalytic reaction). However, when H$_2$O$_2$ concentration is low and there is an adequate hydrogen donator present such as ethanol, methanol, phenol, and others, catalase acts as a peroxide, breaking down H$_2$O$_2$ but oxidizing its substrate (peroxidation reaction).

Superoxide dismutase (cytosolic SOD1) is a homodimeric enzyme with a molecular weight of ~32,000 daltons. It has a copper atom and a zinc atom in each of 16 subunits; these two atoms are anchored to residual histidine number 6120. Subunits are stabilized by an intracatenary disulfide bond associated by non-covalent forces. This enzyme requires copper (Cu) and zinc (Zn) for its biological activity and Cu loss causes its complete inactivation, in many cases leading to disease. This enzyme has great physiological significance and therapeutic potential.

For these reasons and due to the role these enzymes play in oxidative stress regulation, the decision was made to evaluate their expression in CaP and BPH.

METHODS

Study subjects: BPH samples were collected from December 2005 to April 2007 from patients registered in the surgery log of the Urology Service of the Hospital Central Militar that met with inclusion, exclusion and elimination criteria.
CaP samples were collected from tissues obtained from the Pathology storage area of the Hospital Central Militar corresponding to CaP cases from 1999-2003 that met with inclusion, exclusion and elimination criteria.

**Inclusion criteria:** a) patients diagnosed with CaP with lower urinary tract obstruction; b) patients diagnosed with BPH with transurethral resection of the prostate (TURP) and radical prostatectomy indication.

**Exclusion criteria:** a) patients who did not give their authorization to participate in the procedure; b) patients who were not TURP candidates.

**Elimination criteria:** a) insufficient amount of tissue; b) tissue that underwent degradation of genetic material during transfer.

The amount of tissue necessary for the study was from 1-5 g; samples were collected immediately following surgical intervention; maximum transfer time was one hour from the moment of obtaining the samples to their completed transfer to the Molecular Biology Laboratory of the Escuela Médico Militar where they were kept at a temperature of -70°C in a Revco® ultrafreezer (Legaci ULT2186 3-35 Dupont SVVA Refrigerants).

**Immunohistochemistry:** Using a light microscope, CaP and BPH tissue samples were incubated by formalin immersion (pH = 7.4) and embedded in paraffin. Tissue sections (3 μm) were stained with hematoxylin and eosin (H&E) stain. Tissue 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 minutes and washed with distilled water. Slices were incubated with Schiff's reagent for 5 minutes and counterstained with hematoxylin for 30 seconds. Histological profile of 5 randomly selected fields were registered using KS-300 software (Carl Zeiss, Jena, Germany). The percentage of damaged area with histopathological alterations was obtained (400x magnification). For immunohistochemistry, tissue sections (3 μm) were deparaffinized and heated to expose antigenic sites; peroxidase endogenous activity was blocked with 0.03% of H₂O₂ in absolute methanol. Tissue sections were incubated all night at 4°C with a dilution of 1:200 of monoclonal anti-catalase antibody and monoclonal anti-SOD1 antibody in TRIS solution. Primary antibody was removed and sections were washed with TRIS twice. Slices were incubated with a dilution of 1:500 of polyclonal rabbit antibody as secondary antibody and washed twice in TRIS. Bound antibodies were detected with the avidin-biotin complex (Vectastain ABC-kit) and diaminobenzidine (DAB) 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 and in the same run, making immunostaining comparable. All specimens were examined under Axiovert 200M light microscope (Carl Zeiss, Jena, Germany). For automated morphometric analysis, the percentage of positive cells (seen as brown) was determined with a KS-300 3.0 computerized image analyzer (Carl Zeiss, Jena, Germany). This equipment automatically detects positive cells and determines their percentage per field. Five random fields were studied at 100x (1 584 000 m² total area). Results were expressed as percentage.

**Data analysis:** Catalase and SOD1 immunoreactivity data were analyzed with the Student t test and expressed as mean ± SD. CaP and BPH groups were compared and statistically significant difference was considered to exist when P < 0.05. Graph Prisma program version 3.32 was used for statistical test application.

## RESULTS

Biological samples were obtained from a total of 80 patients to carry out immunohistochemistry. Forty patients had histopathological CaP diagnosis and 40 had BPH diagnosis.

In the CaP patient group (40 patients), mean age was 65.3 years and mean preoperative PSA concentration was 11.9 ng/mL. Gleason score was 2 in one case (2%), 3 in one case (2%), 4 in seven cases (18%), 5 in six cases (15%), 6 in six cases (15%), 7 in ten cases (25%), 8 in six cases (15%) and 9 in three cases (8%). Radical prostatectomy was performed in all cases (100%) due to clinically localized cancer diagnosis.

In the BPH patient group (40 patients), mean age was 67.5 years. Mean preoperative PSA concentration was 5.8 ng/mL and transurethral resection of the prostate (TURP) was performed on all 40 patients.

An increase in catalase immunoreactivity in stroma in CaP tissue was observed when compared with this same data in BPH tissue (P = 0.0001). On the other hand, immunoreactivity results of the percentage of marked area per field (400x) showed an increase in CaP (P= 0.0001) (Images 1 and 3, panels A and B). Immunoreactive data in the glands of CaP and BPH tissue were compared and revealed an increase in CaP (P = 0.0012). When immunoreactive marked areas per microscopic field (400x) were compared, an increase in CaP was seen (P = 0.0001) and immunoreactivity...
relation was 2.7:1 (Images 1 and 3, panels C and D). Immunoreactivity of SOD1 antioxidant enzyme in CaP tissue stroma was increased when compared with BPH tissue stroma ($P = 0.0001$). Immunoreactivity results of percentage of marked area per field (400x) showed an increase in CaP tissues ($P = 0.0001$) (Images 2 and 4, panels A and B). SOD1 immunoreactivity data in CaP as well as BPH tissue glands were compared and showed an increase in CaP ($P = 0.0005$) and comparison of glandular tissue of immunoreactive marked areas per microscopic field (400x) showed an increase in CaP ($P = 0.0001$). Immunoreactivity relation was 2.3:1 (Images 2 and 4, panels C and D).

**DISCUSSION**

In the present study SOD1 and catalase expression levels were higher in CaP tissue than in BPH tissue. These findings contradict the immunohistochemical results of Baker et al, who used the same antibodies. They found a decrease in immunoreactivity in these enzymes, whereas the present authors found a significant increase in catalase and SOD1 in both stroma and glandular tissue. Immunoreactivity of these two enzymes increased diffusely in stroma and glandular tissue of the tumors. It is possible that the expression of these enzymes is altered in both tissues due to the oxidative effect or to the increase in oxidative imbalance caused by an increase in free radical production.

There are no standardized methods for measuring the state of overall oxidative stress in humans. In other words, none of the so-called oxidative stress biomarkers are singly capable of providing a precise oxidative stress evaluation that can then be directly applied to clinical practice. Therefore it should be taken into consideration that antioxidant enzyme expression in tissue is only an approximation of the measurement of...
the oxidation-reduction state of said tissue. Antioxidant protein expression is induced at the transcriptional level by oxidative stress. This becomes obvious in the present study with the concentration increase in both enzymes. Thus it can be inferred that the increase in antioxidant enzyme levels in CaP indicates the existence of a response to oxidative stress in those tissue cells.

Oxidative stress is thought to contribute to, or to be a risk factor for, CaP. The present study is congruent with this hypothesis, which is supported by considering oxidative stress to be a common characteristic of various pathological conditions, including CaP. The first of these pathological entities is seen in aging and in various types of cancer. They are associated with a state in which the oxidant-antioxidant balance changes, leading to oxidative stress. The mitochondrion is the greatest intracellular source of ROS and is also vulnerable to oxidative damage that probably produces the problems of human aging. ROS and free radical production in the mitochondrion is increased during aging as a result of the permanent increase in the electron flow of the altered cellular respiratory system. Cellular oxidative damage and the resulting mutations of mitochondrial DNA (mtDNA) can cause an additional decline in cell and tissue functions. The accumulation of somatic mtDNA mutations by means of oxidative stress seems to be the biggest contributor to human aging in relation to degenerative disorders, including prostate disease. Another important condition is exposure to androgens, which has been widely associated with the development of CaP and is a means by which the oxidant/antioxidant balance of histological strains of the prostate are altered. Physiological levels of androgens are capable of increasing oxidative stress in androgen-sensitive cells in CaP. There is evidence suggesting this is partially due to the increase in mitochondrial activity. Androgens also alter intracellular glutathione levels and the activity of certain detoxifying enzymes such as glutathione peroxidase and superoxide dismutase.
as γ-glutamyl transpeptidase, which is important for maintaining the cellular oxidant/antioxidant balance. 14

The effect of androgens on CaP cells includes stimulation of PSA production. The effect of androgens on the creation of ROS is mediated in part by PSA. Other conditions that are related to CaP and that are associated with an increase in ROS generation are smoking, diet and environmental toxins. The increase of these enzymes can be studied and taken into consideration in order to evaluate the progression of this disease, serving as prognostic markers as well as being useful in treatment evaluation in certain patients, thus helping define the therapeutic conduct of the treating physician.

**CONCLUSIONS**

Densitometric expression and the percentage of area marked by the antioxidant enzyme catalase and SOD1 groups is increased in CaP tissue when compared with BPH tissue.

These results strongly suggest the application of SOD1 and catalase as prostate tissue tumor markers. They also indicate that CaP tumor cells are under oxidative stress, which can cause continuous genetic changes resulting in an increase in chromosome abnormalities and in mutations that in turn can favor tumor genesis and propagation.

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*Image 4.* SOD1 immunohistochemical determination in CaP and BPH stroma. In CaP stroma (A), in BPH stroma (B); in both groups (n = 40) densitometric value per field (400x) was determined and values were analyzed (P = 0.0001) with significant increase in immunoreactivity for SOD1 in CaP. SOD1 immunohistochemical determination in CaP gland and BPH gland. In CaP gland (C), in BPH gland (D); in both groups (n = 40) densitometric value per field (400x) was determined and values were analyzed (P = 0.0005) with significant increase in immunoreactivity for SOD1 in CaP.
BIBLIOGRAPHY