ABSTRACT

In addition to its antioxidant function, manganese superoxide dismutase (Mn-SOD) plays a role in tumor suppression in different neoplasms, including prostate tumors. Greater Mn-SOD expression in prostate cancer (CaP) tissue than in benign prostate hyperplasia (BPH) tissue in stromal cells as well as glandular tissue has been demonstrated. Prostate specific antigen (PSA), even levels below 4.0 ng/mL, is known to be a CaP risk marker and only 8% of the cancer-free population presents with higher PSA levels. The present study analyzes Mn-SOD expression levels in CaP tissue and correlates them with PSA levels.

Materials and methods: PSA level was retrieved from case records and Mn-SOD expression levels were determined by immunohistochemistry.

Results: Of the 26 diagnosed cancer tissue samples, no correlation was found between Mn-SOD expression levels and PSA levels. Data was analyzed using Pearson correlation with an index of 0.04182 and p-value was P=0.4163.

Conclusions: There was no correlation between Mn-SOD expression levels and PSA levels in the cancer tissue studied.

RESUMEN

Se ha evidenciado que la dismutasa de manganeso-superoxído (MnSOD), además de su función antioxidante, tiene un papel supresor en diversos tumores, entre ellos el de próstata. Ya se ha demostrado que existe una mayor expresión de la dismutasa de Mn-superoxído en tejidos de cáncer de próstata que en la hiperplasia prostática benigna, tanto en estroma como en tejido glandular. Por otro lado, se sabe que las concentraciones del antígeno prostático específico, incluso por debajo de 4.0 ng/mL, son un marcador de riesgo del cáncer de próstata y que sólo 8% de la población sin datos de cáncer presenta niveles superiores. En este estudio se analizan los niveles de expresión de la dismutasa de Mn-superoxído en tejidos con cáncer de próstata y se correlacionan con las concentraciones de antígeno prostático específico.

Material y métodos: Se revisaron los expedientes clínicos para la obtención del antígeno prostático específico. La expresión de la dismutasa de Mn-superoxído se determinó por inmunohistoquímica.
**INTRODUCTION**

The specific causes determining prostate cancer (CaP) onset and progression are not known. However, genetic and environmental factors are attributed to this disease progression. Individuals with a first-degree relative with CaP have a doubled risk for developing this neoplasia and that risk increases 5-11 times if the number of first-degree relatives is two or three.

Prostate cancer onset and progression are influenced by androgens. A high fat diet promotes prostate cancer cell lines (LNCaP) and fats are a source of free radicals that promote CaP. There are genetic polymorphisms associated with CaP risk such as in the 5α reductase type 2 and 3β-hydroxysteroid-dehydrogenase type 2 genes. The androgen receptor gene presents 2 polymorphisms that play a role in CaP onset and progression. There is a factor localized in the long arm of chromosome 1 (1q24-25) that increases the risk of developing CaP at an early age. Protective factors associated with CaP are vitamin D, a steroid hormone that inhibits LNCaP proliferation and induces its differentiation, vitamin E, a powerful antioxidant cell membrane protector and high doses of vitamin A and lycopene, a carotenoid present in tomatoes.

Prostate cancer is one of the most common malignant tumors worldwide and is the fourth cause of death by cancer in men. In 2004 in the United States, close to 230,000 cases of CaP were diagnosed and 29,900 of them resulted in death. The treatment paradox is that even though the disease is still the second cause of death by cancer in men, the 8:1 ratio between CaP and specific mortality indicates that the large majority of men do not die from the disease.

After the age of fifty, CaP incidence is 3 or 4 times higher every 10 years. African Americans have the highest CaP incidence rate. Worldwide incidence for this group has increased from 124 for every 100,000 male inhabitants to 250 per 100,000, representing a 102% increase. Hispanic Americans have an intermediate incidence rate of 104 per 100,000. Asian countries, especially China and Japan, have the lowest CaP incidence and mortality rates in the world.

In regard to survival rate indexes, 92% of diagnosed patients live at least five years longer and 67% at least ten years longer. Five-year survival index is 100% in localized cancer. The percentage of prostate cancers detected in early stages is 58%. These figures change when the cancer has spread, but if it has done so only locally the 5-year survival rate is 94%. Thirty-one percent of tumors are detected in this condition. The worst prognosis is when tumors are detected in late stages. This happens in 11% of tumors and the consequent 5-year survival rate drops to 31%.

In Mexico CaP is the second most common tumor after skin cancer in men and in 2001 it was the first cause of death from malignant tumor.

Currently there are two tumor staging systems: the TNM (Tumor, Node, Metastasis) system widely used in the European medical community and the American modified Whitmore-Jewett staging system. The TNM system has a larger number of subdivisions.
The TNM staging system was proposed and published in 1978 by the American Joint Committee on Cancer (AJCC) and the Union Internationale Contre le Cancer (UICC). This system evaluates local tumor stage, the degree to which lymph nodes have been affected, and the presence of metastasis. The Gleason system is the most widely used method for analyzing histological differentiation grade. It was proposed by the Veterans Administration Cooperative Urological Research Group (VACURG). Classification is based on the pattern of tumor growth in reference to prostate stroma. This pattern varies from well-differentiated (Grade I) to non-differentiated (Grade V). Due to the fact that the majority of prostate carcinomas contain more than one histological pattern, this system also takes heterogeneity into account in relation to the antioxidant defense system reduction resulting in a greater reactive oxygen species (ROS) concentration.

Oxidative stress is considered to be a state in which there is an excess of ROS as well as a reduction of endogenous antioxidants, thus manifesting lesions produced by free radicals. These free radicals react with lipids, proteins, carbohydrates and cell DNA, but also with components of the extracellular matrix. Therefore they can cause irreversible damage that can lead to apoptosis if it is very extensive.

Oxidant and antioxidant imbalance is associated with the physiology of various pathological entities and the biological aging process is speeded up in direct relation to oxidative stress magnitude.

Oxidative stress has recently been associated with having a critical role in various clinical conditions, including malignant disease. ROS can cause DNA oxidation and protein damage, tumor suppressor gene damage and an increase in proto-oncogene expression. Cancer shows a pro-oxidative change in the oxidoreduction stage.

ROS are potential carcinogens because they facilitate mutagenesis and tumor promotion and progression. Even normal cells show increased proliferation and expression in growth-related genes if they are exposed to hydrogen peroxide or superoxide ($O_2^{\cdot -}$).

The majority of ROS-induced mutations appear to encircle guanine, causing G-T transversions. If this involves critical genes such as oncogenes or tumor suppressing genes it can result in cancer onset or progression. Chronic prostate hyperplasia is diagnosed in the majority of men around 40 years of age. Late appearance of CaP suggests that a multistep process is involved in carcinogenesis and the most logical factor in the endogenous formation of genotoxins in these later stages of life is ROS accumulation.

Superoxide dismutase (SOD) forms a group of enzymes that are present in almost all cells. It catalyzes the reaction of two $O_2^{\cdot -}$ anions to form hydrogen peroxide.

Only anaerobic organisms or a few aerobic bacteria lack SOD since its physiological function is to eliminate $O_2^{\cdot -}$ produced in reactions of the aerobic metabolism. Depending on the metal used as a cofactor, there are three forms of SOD. These three forms can be divided into two different phylogenetic families: CuZnSODs and Fe/Mn-SODs.

Mn-SOD belongs to a metalloprotein family whose function is to catalyze and remove toxic superoxide anions. Mn-SOD is a 96 kDa tetramer that contains a manganese ion and is found in the mitochondrial matrix. Mn-SOD is an enzyme that is able to be induced by tumor necrosis factor (TNF) that protects cells from apoptosis mediated by TNF through $O_2^{\cdot -}$ detoxification, subsequent apoptosis regulation through Cytokine and modulation of the redox stage of the mitochondrion. Mn-SOD has been shown to be a tumor suppressor in breast cancer. Overexpression of this enzyme protects neurons from N-methyl-D-aspartic acid (NMDA) and toxicity induced by nitric oxide. Numerous studies have indicated that Mn-SOD plays an important role in inhibiting tumorigenicity.

Mn-SOD plays a dominant role in cellular protection against oxidative damage and cellular concentration of $O_2^{\cdot -}$ regulation, which is extremely oxidizing and an undesirable by-product of cellular metabolism. Mn-SOD level alterations have been associated with a number of neurodegenerative illnesses including Parkinson's disease, Duchenne muscular dystrophy, Charcot-Marie-Tooth disease and Kennedy's disease.

Low Mn-SOD activity has been demonstrated in many different types of tumor. Mn-SOD overexpression suppresses tumorigenicity in human melanoma, breast cancer cells and glioma cells, suggesting that Mn-SOD is a tumor suppressing gene in a wide variety of cancers. For example, St. Clair and others stated that Mn-SOD tumor metastasis suppression is associated with the reduction in reduced tumorigenicity and fibronectin elevation. They found that the average periods of tumor growth for tumors derived from all transferred Mn-SOD cells were longer than those of the parental cells. They also found elevated levels of extracellular matrix fibronectin in varieties of transferred Mn-SOD. Oxidative stress can inactivate the promoter of the human
fibronectin gene that contains the obligatory sites of the SP-1 protein and antioxidants that support the activity of SP-1 protein transcription. These researchers speculate that Mn-SOD expression provides an antioxidant environment that supports SP-1 activity, leading to an increased level of fibrogen in transferred Mn-SOD cells. The most promising role of Mn-SOD is associated with its inhibition of tumorigenicity. Many studies have suggested that Mn-SOD can act as a general tumor suppressing gene. It is believed that Mn-SOD will be used in cancer therapy in the near future.  

In relation to other cancers, Mn-SOD plays a suppressing role in ovarian cancer, breast cancer, lung cancer and possibly prostate cancer.  

Prostate specific antigen (PSA) is a one-chain glycoprotein (33kDa). PSA is produced and secreted by the prostatic epithelium and is one of the most abundant seminal plasma proteins, where it has been found at concentrations of 0.2-5.0 mg/mL.

Normal serum PSA range is 0.1-4.0 ng/mL. In addition to the prostate, PSA production has also been detected in the periurethral glands. PSA contributes to the semen liquefaction process through semenogelin protein hydrolysis.  

Even though PSA is more abundant in semen, a small amount is also found in blood, usually at very low levels defined in the range of 0-4.0 ng per milliliter. Normal range for the first commercial PSA test (Tandem-R PSA) created by Hybritech in 1986 was established based on a study showing that 99% of 472 apparently healthy men had a total PSA under 4 ng/mL. The cut-off point of normal levels can increase, depending on the age of the patient. Therefore, a 4 ng/mL serum PSA level may be considered elevated in a 50-year-old man and normal in an 80-year-old. PSA levels have a 15% random variation in the same individual. If a PSA is first reported as 3 ng/mL and then is reported as 3.5 or 2.5 ng/mL when the test is repeated on a different occasion, it would be considered normal. PSA levels in hospitalized patients may diminish by 50%. The majority of men have PSA levels under 4 nanograms per milliliter of blood. Serum PSA level is presently the most sensitive test for early detection of CaP since it is elevated in approximately 65% of cases.  

When CaP develops, PSA levels are above 4. If levels are between 4 and 10 there is a 25% probability of CaP. If PSA levels are above 10 that probability increases to 67% and continues to increase as PSA levels go up. PSA is an imperfect tumor marker due to its lack of specificity since it can be affected by many factors. PSA elevation in plasma is proportional to tumor mass and therefore PSA in blood is an excellent test for detecting CaP. PSA values tend to be higher the more advanced a tumor is but this is not always the case. A certain percentage of CaP patients have normal PSA levels and so their results give a false negative.  

PSA is present in the blood in two main forms. The majority circulates in the blood surrounded by and bound to plasmatic proteins and a small quantity circulates free from protein binds and is called «free PSA». The free PSA percentage test indicates the quantity of total PSA circulating freely compared with that which is bound to proteins. The risk of cancer increases if the relation between free PSA and total PSA is free less than 10%. This means the lower the proportion, the greater the probability of presenting with CaP. For example, if total PSA is between 4-10 ng/mL, an abnormal value with a calculated 25% CaP risk, and at the same time a low percentage of the total PSA is free PSA (less than 10%) the probability of CaP will increase to 50%, making diagnostic biopsy necessary. A recent study found that prostate biopsy in men with PSA results in the upper limit (4-10 ng/mL) was only justified when free PSA percentage was less than 25%, and so there had been unnecessary biopsy of 20%. It now appears that measuring the relation of free PSA with the total is of particular interest in eliminating unnecessary biopsy in men with PSA levels between 4 and 10.  

Due to the fact that PSA is a specific tissue marker as opposed to a tumor marker, an overlapping of serum levels corresponding to patients with benign prostatic hyperplasia and organ-confined cancer has been observed. Between 38-48% of patients with organ-confined cancer have serum PSA levels within the normal range. Elevated PSA levels have been found in other prostatic pathologies such as prostatitis, benign hypertrophy and prostatic infant. An increase in serum PSA levels due to surgical manipulation has also been observed. This limitation underlines the necessity of studying prostatic pathology from a molecular perspective for the purpose of profiling and/ or complementing today's diagnostic methods with new markers.  

**MATERIALS AND METHODS**  

**Study subjects**: Tissue corresponding to patients diagnosed with CaP between the years 1999-2003 and stored at the Department of Pathology of the Hospital Central Militar was used. The respective case records were reviewed as well as the Pathology Department surgical books in order to fill out the data recollection form.  

To obtain samples, tissues with cancer meeting inclusion, exclusion and elimination criteria were
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requested from the Pathology Department of the Hospital Central Militar.

1. Inclusion criteria: Tissue from patients histopathologically diagnosed with CaP obtained from the storage area of the Pathology Department that had been registered between the years 1999-2003.

2. Exclusion criteria: Tissue from patients diagnosed with cancer that had been obtained through surgical resection in which there were no cancer foci.

3. Elimination criteria: (a) Tissue that had suffered adulteration of genetic material and (b) Tissue with cancer foci under 1cm².

Immunohistochemistry: CaP and BPH tissue were fixed by formalin immersion (pH=7.4) and embedded in paraffin. For histological analysis, tissue sections (3 μm) were stained with hematoxylin and eosin (H&E). Tissue sections were stained with periodic acid-Shiff (PAS) to show polysaccharides, mucopolysaccharides and glycoproteins of the cellular membrane. Slices were incubated with periodic acid for 5 minutes and washed with distilled water and then incubated with the Shiff reactive for 5 minutes and counter-stained with hematoxylin for 30 seconds. The histological profile of the 5 randomly selected fields were registered with KS-300 software (Carl Zeiss, Jena, Germany). The percentage of damaged area with histopathological alterations was obtained (400X magnification). To carry out immunohistochemistry, paraffin was removed from the tissue sections (3 mm) and they were heated to unmask antigen sites. Endogenous peroxidase activity was blocked with 0.03% H₂O₂ in absolute methanol. Tissue sections were incubated overnight in Tris solution at 4ºC at a 1:200 dilution of monoclonal antibody to Mn-SOD. Primary antibody was removed and 2 repetitive washouts with Tris were done. Slices were incubated with a 1:500 dilution of rabbit polyclonal antibody as secondary antibody and 2 repetitive washouts with Tris were done. Bound antibodies were detected with avidin-biotin complex (Vectastain ABC-kit) and diaminobenzidine as substrate. After repeated washout with Tris, slices were counter-stained with hematoxylin. All slices were incubated at the same time, under the same conditions and at the same antibody concentration, so immunostaining was comparable. All specimens were examined using an Axiovert 200M inverted microscope (Carl Zeiss, Jena, Germany). To make the automatized morphometric analysis, the percentage of positive cells (brown color) 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 (total area 1, 584,000 m²). Results were expressed as percentages.

Images were analyzed using KS300 software; three images per structure were taken (epithelial, bronchial and alveolar, where mark was found).

Statistical analysis: The correlation between Mn-SOD immunoreactivity and PSA serum levels was analyzed with the Pearson correlation test and data were expressed with mean standard deviation. Statistical significance was considered to exist when P < 0.05.

RESULTS

General data In the present study patients recorded in the surgery registrar of the Urology section of the Hospital Central Militar that fit the inclusion criteria were taken into account.

Biological samples had been obtained from 26 patients clinically and histopathologically diagnosed with CaP.

CaP patient age was 68.8 ± 48.1, representing a median of 69, mode of 74, minimum age of 55 years and maximum age of 80 years (image 1).

Gleason score of the study group was 4.47, with median value of 5 and maximum value of 9.

Study group prostate antigen value was 35.29, with median value of 14.07, minimum value of 0.01 and maximum value of 41.26.

Correlation between PSA levels and CaP tissue immunoreactivity. No correlation was found between Mn-SOD expression levels and serum PSA levels using the Pearson correlation test. Therefore the null hypothesis was rejected and the alternate hypothesis was accepted.

Mn-SOD expression in tissue studied In relation to Mn-SOD immunoreactivity in the tissue studied, densitometric values were in the range of 90.7901-135.8088 and did not correlate with PSA values (Images 2 and 3).

In certain patients these data may possibly be applied to evaluate the degree of disease aggressiveness according to densitometric values established in CaP patient biopsy. These values could serve as a guide in certain treatments for evaluating treatment progression through biopsy analysis.
Prostate specific antigen (PSA) is a prostate-exclusive protein substance whose function is to dissolve seminal clots. Normal PSA blood levels in healthy men are very low, in the order of millions of times less than in semen and they increase in the presence of different prostate diseases.28,29 In the present study PSA level concentrations were from 0.01 - 41.26.

Serum PSA concentrations are directly proportional to transitional zone volume, a histologically well-defined zone that is enlarged in the majority of CaP patients. In previous studies by this study’s same authors there was Mn-SOD overexpression in CaP when compared with BPH tissue (data not published). In relation to those findings, data from the present study are consistent with those of the literature where various authors have described an increase in Mn-SOD expression in certain tumors and that PSA levels above and even below 4 are a CaP risk marker.14,30,31 In 2005, W.F.Tohn et al. stated that PSA levels of 10 indicated high incidence risk for CaP. However, they found that it was not important to measure PSA density. In 2005, Ian M. Thompson et al. stated that even though PSA measurement has led to greater cancer detection at a smaller tumor phase it is still not known if PSA screening significantly reduces CaP mortality. This author suggests that due to the great variability between serum PSA levels and cancer diagnosis, terms such as normal and elevated in relation to PSA measurement in cancer should be used with caution. There is not enough solid and consistent evidence to absolutely determine the correct figures, given that 8% of the population can have values above 4ng/mL and not present with CaP.

In relation to Mn-SOD expression certain studies state that this enzyme can function as a tumor suppressor, possibly due to apoptotic modulation of cell growth and proliferation.32 This enzyme converts reactive oxygen species (ROS) to oxygen and hydrogen peroxide and the latter is catalyzed in water by catalase and peroxidase glutathione, a selenium-dependent enzyme.6 It is known that Mn-SOD concentration is increased in tumors such as ovarian, pleura, stomach and esophageal tumors.14,30,31 Previous studies show (data not published) Mn-SOD overexpression in CaP when compared with BPH. The present study data concur with those of the prior studies and there was no correlation between Mn-SOD overexpression and serum PSA levels.

Twenty-six tissues with histopathological CaP diagnosis were analyzed in the present study. Figure 1 shows there was no correlation between Mn-SOD expression and serum PSA levels and this is consistent with data described in the literature. In 2004 Kinnula et al. reported that there was Mn-SOD overexpression in pleura mesothelioma, one of the most aggressive human tumors. In 1998 Kahlos et al. stated that Mn-
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SOD expression in pleural mesothelial cells was low compared with that in malignant pathology in which its expression was increased. On the other hand, in 2005 Hu et al. reported that Mn-SOD levels in CaP tissue were unchanged and even diminished. In the present study there was no correlation between Mn-SOD and PSA levels but a homogeneous tendency toward overexpression of this enzyme was observed. This can be seen in Figure 1, where Mn-SOD densitometric values were distributed between 90.7901 and 135.8088.

There is great interest in regard to the function of oxygen free radicals in CaP incidence, progression or mortality. In previous studies Mn-SOD overexpression in CaP tumor tissue was observed, suggesting that enzymatic antioxidant mechanisms are altered by an excessive creation of free radicals, as previously mentioned. Apparently, presumably high levels of free radicals in general are not correlated with PSA levels.

A large part of the research that has shown a relation between ROS and CaP has been epidemiologic in nature, mainly cohort and case and control studies. Prospective studies are in the making. The results registered here directly relate antioxidant enzyme levels with CaP, since Mn-SOD overexpression was found in the different tissues studied. Chronic inflammation has been linked to the incidence of many cancers including CaP. Research studies with human tissue samples have shown that epithelial cell proliferation is the result of inflammation increase. Sikka et al. found a cancer risk increase associated with inflammatory mechanisms since 15% of all cancers may be related to chronic inflammation. Epidemiological studies have found a greater prostate cancer risk in men with a prior history of sexually transmitted diseases or prostatitis. Proliferative inflammatory atrophy has recently been proposed as a precursor of prostatic intraepithelial neoplasia and CaP. Inflammation can lead to oxidative stress persistence in cancerous cells and ROS can represent a survival advantage for them.

Elevated oxidative stress levels in cancer can stimulate cellular proliferation and increase DNA mutation rates through epigenetic damage and/or changes. In their studies, Zentella, Saldaña et al. showed that antioxidant enzyme loss as an early event in prostate tumors established the basis for ROS growth stimulation. Oxidative stress accentuates cancer cell proliferation through an increase in growth factor receptor sensitivity and by altering transcription factor activity. Inflammatory cells such as macrophages and mast cells release angiogenic and cytosine factors such as alpha tumor necrosis factor, interleukine-1, and vascular endothelial growth factor which signal cell growth and proliferation. In addition, cytosines regulate signaling pathways that control proliferation, apoptosis, differentiation and metastasis.

Oxidative stress is thought to be associated with many human diseases either as cause or as effect. Different diseases, including cancer, cardiovascular disease and diabetes mellitus are associated with oxidative damage presumably mediated by means of reactive oxygen and nitrogen species. Lipids are the principal cellular components that are sensitive to damage caused by free radicals (peroxidation of membrane fatty acids), proteins (denaturalization) and nucleic acids. The role of antioxidants in protecting against oxidative stress and the development and progression of cancer continues to be controversial.

Mn-SOD is an antioxidant enzyme that catalyzes $O_2^-$ conversion to less reactive ROS, although studies reporting its role in cancer development and progression are relatively few and still controversial. In human and murine breast cancer models and in skin cancer models antioxidant agents showed a significant reduction in tumor incidence. Despite a large quantity of assays that are available for measuring oxidative stress and antioxidant state, there are currently no methods for estimating oxidative stress, making its measurements somewhat arbitrary.
From a practical point of view, a desired result is preventing disease from progressing to a metastatic stage in which there is an increase in cancer-related mortality risk. This objective can be reached not only through disease elimination but also by halting tumor growth. Whether serum PSA levels represent a clinically valid sign is still controversial.

In the present study Mn-SOD in glandular tissue was neither diminished nor increased in relation to PSA concentration. Therefore there was no increase in levels considered to be normal and Mn-SOD immunoreactivity was not correlated with serum PSA levels. Since CaP etiopathogenesis is related to oxidative stress and since Mn-SOD is one of the main antioxidant enzymes, its increase in cancer tissue suggests that this enzyme carries out an important function in tissues with increased oxidative stress.\(^\text{13}\)

There are discrepancies in the literature with respect to the importance of Mn-SOD in CaP since its presence can mean various things: an increase in normal values may be the result of organism compensation when there is an increase of oxygen free radicals in the tissue studied and/or this increase in free radicals may be due to the reduction of antioxidant systems. Both situations may even be occurring in the tissues. Further research will reveal the function of antioxidant enzymes, including Mn-SOD, in prostate cancer.\(^\text{25,21,29,36,37}\)

**CONCLUSIONS**

There was no correlation between Mn-SOD expression levels and serum PSA values in the cancer tissue studied. In relation to the Mn-SOD immunoreactivity of these tissues, densitometric values ranged from 90.7901-135.8088 and were not correlated with PSA values. The data of the present study may be used in evaluating the degree of disease aggressiveness in certain patients according to densitometric values established through CaP patient biopsy. These values could also be a guide in certain treatments for evaluating treatment progression through biopsy analysis.

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