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Tumoral Markers in Prostate Cancer

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1. Introduction

In Mexico, in 70% of cases, the prostate cancer (PCa) is found in advanced stage. PCa currently occupies second place in frequency of cancer in men, surpassed only by skin cancer, and is the second principal cause of death in men after of lung cancer (Hall et al., 2005).

Reactive oxygen species (ROS) such as superoxide ($O_2^{\bullet-}$) and hydrogen peroxide (H_2O_2) are found in a large number of tumors and in high levels they induce cell death, apoptosis, senescence and angiogenesis (Ushio-Fukai & Nakamura, 2008).

One of the major sources of ROS is NADPH oxidase (NOX). The NOX are a family of enzymes that are found in various tissues. The NOX receives an electron from NADPH generating $O_2^{\bullet-}$ (Bánfi et al., 2001). Xia et al, Lim et al. and Brar et al. found that some NOX isozymes increase in association with ROS-production and tumor progression in ovarian and human colon cancer and in DU-145 cells of PCa, respectively (Brar et al., 2003; Lim et al., 2005; Xia et al., 2007).

Cells have different antioxidant systems including low molecular weight antioxidant molecules and various antioxidant enzymes. Superoxide dismutase (SOD) catalyses the dismutation of $O_2^{\bullet-}$ into H_2O_2 that can be transformed into H_2O and O_2 by catalase (CAT) (Genkinger et al., 2006). Mn-SOD is the major antioxidant in the mitochondria and is essential to the vitality of mammalian cells. In many types of tumor cells has been found to contain high levels of Mn-SOD, Cu/Zn-SOD or CAT expression compared to their nonmalignant counterpart such as in human tumor cancer cells of esophageal, gastric, ovary, breast, neuroblastoma, osteosarcoma, melanoma, pleura and leukemia (Grigolo et al., 1998; Janssen et al., 2000; Starcevic et al., 2003; Qian et al., 2005, López Laur et al., 2008). However, the role of these enzymes in carcinogenesis remains unclear.

On the other hand, iNOS or NOS-2 is an inducible isoform of nitric oxide synthases (NOS). All isoforms of NOS catalyze the reaction of L-arginine, NADPH and oxygen to nitric oxide ($NO\bullet$), L-citrulline and NADP. $NO\bullet$ is a lipophilic physiological messenger which regulate a variety of cellular responses and may exert its cellular action by cGMP-dependent as well as by cGMP-independent pathways (Stamler, 1994). The expression of iNOS has been found to be increased in a variety of human cancers such as colon, stomach, brain and breasts cancers (Alderton et al., 2001; Church & Fulton, 2006) by multiple mechanisms that control their activity (Stamler, 1994; Friebe & Koesling, 2003).

Cyclooxygenase-1 and 2 (COX-1/2) catalyze the initial step in the formation of prostaglandins (Smith & Langenbach, 2001). Very recently their role in carcinogenesis has become more evident. They influence apoptosis, angiogenesis, and invasion, and play a key role in the production of carcinogens. Usually, a high level of COX expression is found in cancer cells (Dannenberg & Zakim, 1999). The role of COX-2 in carcinogenesis has been recently described. Multiple lines of evidence confirm that selective COX-2 inhibitors reduce prostaglandin production and the risk of colorectal, skin and other neoplasias (Sonoshita et al., 2001). COX-2 is related to the formation of carcinogens, tumor promotion and inhibition of apoptosis, angiogenesis and the metastatic process (Ebehart et al., 1994; Uefuji et al., 2000). However, the interactions and links between lipid metabolism and cancer progression remain to be elucidated.

Therefore, in the present study, we decided to evaluate and compare, for the first time, the pattern protein expression of p22 *phox* subunit of NOX, Mn-SOD, Cu/Zn-SOD, CAT, iNOS and COX-2 protein expression in patients with PCa and with BPH.

2. Patients and methods

We obtained 62 samples of prostate tissue through of various surgical procedures (transurethral resection and biopsy transrectal). Approval was obtained from the local research and ethics committee for use of tissue. Of these samples, 30 patients (48.4%) had a diagnosis of PCa, while as 32 patients (51.6%) had a diagnosis of BPH (Department of Medical Urology, Hospital Central Militar, Mexico). The sample collection was conducted from January 2006 to December 2009 and was considered inclusion, exclusion and elimination criteria.

In the PCa group the average age was of 65.3 years and the concentration of preoperative PSA was of 8.6 ng/mL. In this group, the patients were classified according to Gleason scale. The score was of 4 in 1 case (3.3%), 6 in 19 cases (63.3%), 7 in 9 cases (30%) and 8 in 1 case (3.3%). None of the patients had undergone chemotherapy or radiotherapy before surgery.

In the BPH group the average age was of 66.5 years and the concentration of preoperative PSA was of 8.7 ng/mL.

Tissues obtained (500 mg) were stored at -83°C (Revco® Legaci ULT2186 3-35 Dupont SVVA Refrigerants) until further processing.

2.1 Immunohistochemistry

For light microscopy, tissue samples of PCa and BPH were fixed by immersion in formalin (pH 7.4) and embedded in paraffin. Serial cuts of 3 mm of thickness were mounted on poly-L-lisina coated slides (Sigma, St Louis, MO). Sections were initially deparaffinized by washing in xylene and decreasing ethanol concentrations and boiled in Declere (Cell Marque, Hot Springs, AR) to unmask antigen sites. Slides were washed in phosphate buffer saline (PBS). Endogenous peroxidase activity was blocked by exposing slides to 0.6% H₂O₂ in PBS for 30 min.

After of washing in PBS, nonspecific binding was avoided by incubation with 5% blocking solution (5% normal goat serum in PBS) for 20 min. Sections were incubated overnight (16 h) with primary anti-p22 *phox* subunit NOX, anti-Mn-SOD, anti-Cu/Zn-SOD, anti-CAT, anti-iNOS and anti-COX-2 antibody (1:100 for each one). Following removal of the antibodies and repetitive rinsing with PBS, slides were incubated with a biotinylated goat anti-IgG secondary antibodies (1:500 for each one) (Jackson ImmunoResearch, West Grove, PA). Immunocytochemical identification of positive cells was performed by the use of an avidin-

biotinylated peroxidase complex (ABC-kit Vectastain, Vector Laboratories, Burlingame, CA) and diaminobenzidine (Vector Laboratories, Burlingame, CA). After of intensive washing in PBS, slides were counterstained with hematoxylin. Sections were dehydrated in graded alcohols, treated with xylene and subsequently mounted. All specimens were examined by light microscopy (Axiovert 200 M, Carl Zeiss, Germany), photographs were taken with a digital camera (Axiocam HRC, Carl Zeiss, Germany). The number of positive cells (brown) was determined with a computerized image analyzer KS-300 3.0 (Carl Zeiss, Germany). The percentage of damaged area with histopathological alterations was obtained (400x magnification). Five random fields were studied (total area 1,584,000 μ^2). The results were expressed as a percentage.

2.2 Statistics

Findings were expressed as the mean \pm SD. The statistical significance of the protein expression levels of p22 *phox* subunit of NOX, Mn-SOD, Cu/Zn-SOD, CAT, iNOS and COX-2 between PCa and BPH groups glands or stroma, was determined using the software Prism version 3.32 (GraphPad Prism 4.0 Software, San Diego, CA, USA) with “student t-test”. It was considered a $p < 0.05$ as statistical difference between groups.

3. Results

The results obtained in PCa and BPH groups are summarized in Table 1. NOX, Mn-SOD, Cu/Zn-SOD and CAT protein immunohistochemistry were significantly higher (1.76, 1.7, 1.78 and 5.88 fold, respectively) in stroma and were significantly higher (3.74, 1.69, 4.76 and 1.59 fold ,respectively) in gland of patients with PCa than that in patients with BPH. Moreover, NOX, Mn-SOD and CAT protein expressions were significantly higher in gland than in stroma, while as Cu/Zn-SOD protein expression was significantly higher in stroma than in gland in patients with BPH. NOX and Mn-SOD protein expression were significantly higher in gland than in stroma in patients with PCa. However, iNOS and COX-2 protein expressions were significantly higher in stroma and gland of BPH (1.47 and 2.9 fold, respectively) in comparison with PCa.

Parameters	BPH (n=32)	Gland	PCa (n=30)	Gland
	Stroma		Stroma	
NOX	4.8 \pm 1.9	6.7 \pm 2 ^a	8.45 \pm 1.7 ^c	25.08 \pm 3.5 ^{d,b}
Mn-SOD	11.97 \pm 1.6	14.73 \pm 1.4 ^c	20.45 \pm 2.1 ^c	24.83 \pm 1.7 ^{d,b}
Cu/Zn-SOD	30.3 \pm 6.6 ^d	11.1 \pm 1.9	54.1 \pm 14.6 ^c	52.8 \pm 8.8 ^d
CAT	9.8 \pm 1.5	37.9 \pm 4.5 ^c	57.6 \pm 15.5 ^c	60.1 \pm 4.5 ^d
iNOS	23.3 \pm 8.8 ^e	24.6 \pm 6.3 ^f	15.9 \pm 7.1	16.3 \pm 4.6
COX-2	12.1 \pm 1.3 ^g	14.8 \pm 2.1 ^h	7.4 \pm 0.9	5.12 \pm 0.7

^aP=0.0002 vs stroma BPH; ^bP<0.0001 vs stroma PCa; ^cP<0.0001 vs stroma BPH; ^dP<0.0001 vs gland BPH; ^eP=0.0072 vs stroma PCa; ^fP=0.0016 vs gland PCa; ^gP=0.0314 vs stroma PCa; ^hP=0.0072 vs gland PCa

Table 1. Mean \pm SD NOX, Mn-SOD, Cu/Zn-SOD, CAT, iNOS and COX-2 protein expressions (%) in PCa and BPH group.

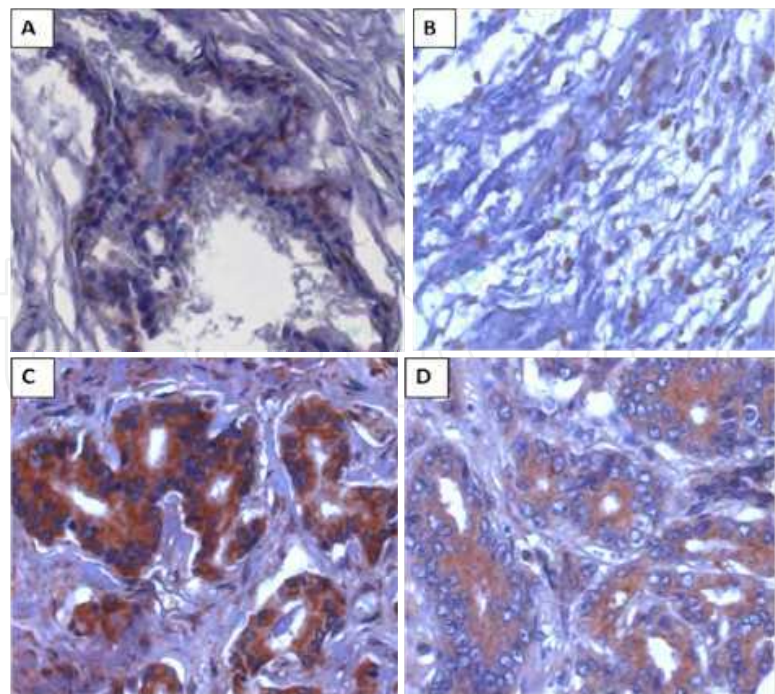


Fig. 1. Immunohistochemical determination of p22 phox subunit of NOX and Mn-SOD in BPH and PCa. (A) y (B) gland of BPH of NOX and Mn-SOD. (C) y (D) gland of PCa of NOX and Mn-SOD. In both groups was determined % area marked by field (400x) and was analyzed the values with significative increase in gland PCa immunoreactivity.

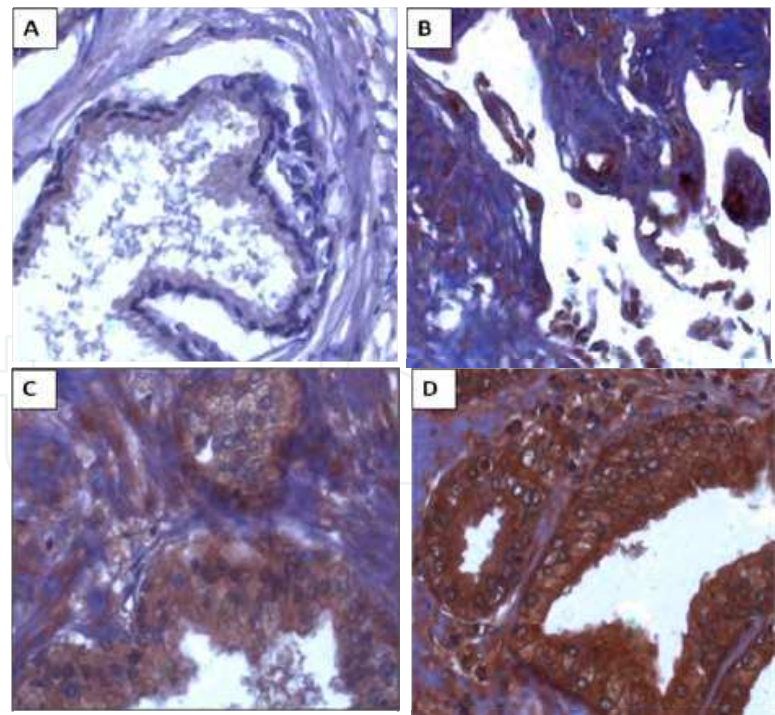


Fig. 2. Immunohistochemical determination of Cu/Zn-SOD and CAT in BPH and PCa. (A) y (B) gland of BPH of Cu/Zn-SOD and CAT. (C) y (D) gland of PCa of Cu/Zn-SOD and CAT. In both groups was determined % area marked by field (400x) and was analyzed the values with significative increase in gland PCa immunoreactivity.

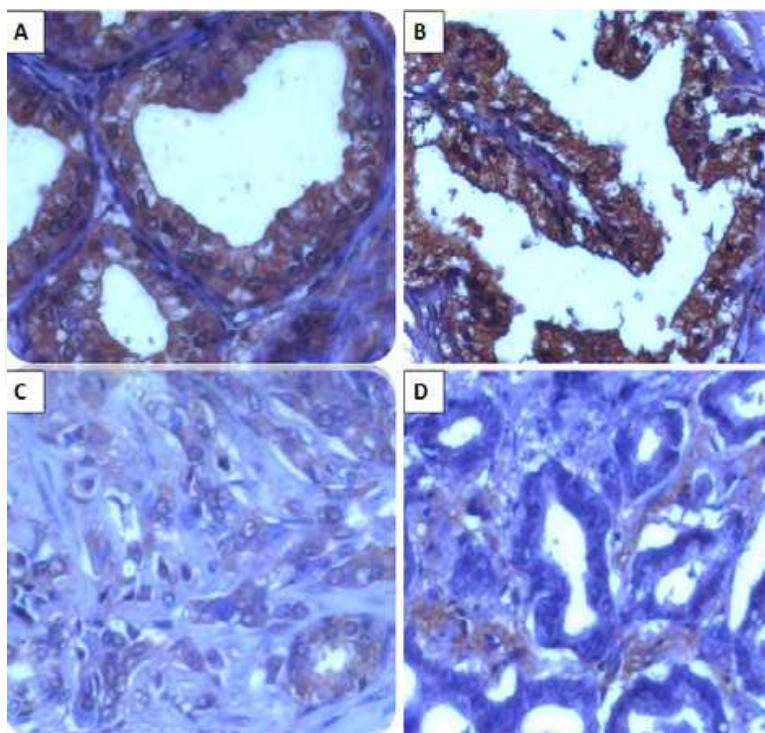


Fig. 3. Immunohistochemical determination of iNOS and COX-2 in BPH and PCa. (A) y (B) gland of BPH of iNOS and COX-2. (C) y (D) gland of PCa iNOS and COX-2. In both groups was determined % area marked by field (400x) and was analyzed the values with significant increase in gland PCa immunoreactivity.

4. Discussion

Recently, a new hypothesis has been proposed for prostate carcinogenesis. It suggested that exposure to environmental factors such as infectious agents and dietary carcinogens, and hormonal imbalances lead to injury of the prostate and to the development of chronic inflammation and regenerative 'risk factor' lesions, referred to as proliferative inflammatory atrophy (PIA). PCa is associated with oxidative stress, which stimulates the production of reactive oxidative species (ROS) and reactive nitrogen species. Oxidative stress derived from endogenous and exogenous sources are associated with DNA damage that occurs with aging and plays a role in carcinogenesis (Klein et al., 2006).

The results obtained, for the first time, in this study showed an increased in the expression of p22 *phox* subunit of NOX, Mn and Cu/Zn-SOD and CAT in stroma and gland of PCa.

In previous studies concluded that NOX has a role as a signaling mechanism that regulates the cell growth and apoptosis in PCa (Vignais, 2002). The exact signaling pathways of NOX are uncertain and may be tissue specific.

Angiotensin II stimulates the activity of NOX in vascular smooth muscle via protein kinase and NF- κ B in the airways and in melanomas (Arnold et al., 2001). Arbiser et al. demonstrated that NOX-1-induced vascular endothelial growth factor (VEGF) and VEGF receptor expression promoting the angiogenesis and rapid expansion of the tumors (Arbiser et al., 2002). Babior BM found that the high levels of ROS are produced spontaneously in PCa and in ovarian cancer. This high production of reactive species was inhibited using an inhibitor of NOX, the diphenyl iodonium (DPI) and the inhibitor of mitochondrial electron

chain, rotenone (Babior, 1999). This suggest that NOX could promote angiogenesis in the early stages of PCa.

By controlling $O_2^{\bullet-}/H_2O_2$ levels, SOD appears to be a critical enzyme in cancer progression. Bravard et al and St Clair et al suggested to the Mn-SOD as a potential tumoral suppressor that might also be involved in cellular differentiation (Zhao et al., 2001).

We suggested that any mutation or epigenetic changes in Mn-SOD gene are the cause of the high level found in the Mn-SOD expression in PCa in the mitochondria. This could have potential effects on survival and proliferation of tumor cells, a fact which has been found in other tumors with aggressive behavior and with a poor prognosis for the patient.

MnSOD polymorphisms have been investigated in several types of malignancies, such as lung, breast and skin cancer (Liu et al., 2004; Han et al., 2007; Bewick et al., 2008). There are at least two functional validated single nucleotide polymorphisms in Mn-SOD. One of these variants is a change in the amino acid codon 9 from valine (GTT) to alanine (GCT) and another is a change in the amino acid codon 16 from valine (GTT) to alanine (GCT) (Tugcu et al., 2007). These changes alter the secondary structure of the protein, affect the transport of the enzyme into mitochondria and reduce the enzymatic activity of Mn-SOD, leaving the cell vulnerable to oxidative damage.

Our results suggest that Mn-SOD probably plays an important role in resistance to treatment of various tumors or in the evolution of invasive tumors.

Brown et al demonstrated an essential role of $O_2^{\bullet-}$ in the posttranslational activation of Cu/Zn-SOD and in the ratio of active to inactive Cu/Zn-SOD, which may be relevant to various diseases, including cancer (Brown et al., 2004). Therefore, $O_2^{\bullet-}$ production by NOX could be induce protein over-expression of Cu/Zn-SOD in PCa.

CAT plays an integral role in the primary defense against oxidative stress by converting H_2O_2 into H_2O and O_2 . Genetic polymorphisms of CAT can change expression levels of the protein. A $-262C \rightarrow T$ polymorphism in the promoter region of the CAT gene is associated with risk of several conditions related to oxidative stress. A transcription factor binding site search indicates that the -262 C allele is located in close proximity to several binding sites for transcription factors and could potentially influence rates of transcription. Forsberg et al. previously showed that the T allele was associated with greater CAT protein levels in some tissues than the C allele (Forsberg et al., 2001). However, different regulatory mechanism of CAT in PCa should be explained.

Our results showed different expressions in NOX, Mn-SOD, Cu/Zn-SOD and CAT in stroma and gland in PCa and BPH groups. The increase of NOX and Mn-SOD expression in gland of PCa and BPH group may have been due to excessive $O_2^{\bullet-}$ and H_2O_2 production that stimulate migration, invasion and angiogenesis of the tumor cells in response to the intracellular changes in ROS levels in this prostate component. The differences in the architecture of the prostate are most likely related to changes in the tumor invasion process

Furthermore our results suggest that exist alterations in the prooxidative-antioxidative balance in PCa, this imbalance is known to alter cellular redox processes, growth, and proliferation and cell cycles, since it is known that certain free radicals mediate the activation of cellular transduction, of transcription factors such as Fos, Jun and nuclear factor kB and an increase in mitochondrial activity in the cells. Moreover, transcription factors such as Rac1, Ref-1 and p53 regulated by ROS are involved in angiogenesis (Ushio-Fukai & Nakayama, 2008).

NO• is synthesized by three differentially gene-encoded NOS in mammals: neuronal NOS (nNOS or NOS-1), inducible NOS (iNOS or NOS-2) and endothelial NOS (eNOS or NOS-3). All three isoforms present similar structures and catalytic modes. The expression of NOS-2 is induced by inflammatory stimuli while NOS-1 and NOS-3 are more or less constitutively expressed. The active form of NOS-1 and -3 requires two NOS monomers associated with two Ca^{2+} -binding protein calmodulin and cofactors such as (6R)-5,6,7,8-tetrahydrobiopterin (BH_4), FAD, FMN and haem group and catalyze the reaction of L-arginine, NADPH and oxygen to NO•, L-citrulline and NADP (Alderton et al., 2001; Stuehr et al., 2004). NOS isoforms are differentially regulated at transcriptional, translational and post-translational levels. The intracellular localization is relevant for NOS activity. Evidence indicates that NOS are present in plasma membrane, Golgi, cytosol, nucleus and mitochondria (Oess et al., 2006; Iwakiri et al., 2006). The expression of iNOS can be transcriptionally regulated by factors such as cytokines (e.g. interferon- γ (IFN- γ), interleukin-1 β (IL-1 β) and tumour necrosis factor- α (TNF- α), bacterial endotoxin (LPS) and oxidative stress (e.g. under conditions encountered during hypoxia)(Xu & Liu, 1998).

An initial study on iNOS expression in human breast cancer suggested that iNOS activity was higher in less differentiated tumours in a panel of 15 invasive breast carcinomas (Thomsen et al., 1995). Reveneau et al reported NOS activity in 27 of 40 tumours studied (Reveneau et al., 1999). Vakkala et al showed that carcinomas with both iNOS positive tumour and stromal cells had a higher apoptotic index and a higher calculated microvessel density index (Vakkala et al., 2000). Loibl et al further demonstrated that while none of the benign lesions were positive for iNOS, 67% *in situ* carcinomas and 61% invasive lesions showed iNOS tumour cell staining (Loibl et al., 2002).

In addition to breast cancer, iNOS has also been shown to be markedly expressed in approximately 60% of human adenomas and in 20-25% of colon carcinomas, while expression was either low or absent in the surrounding normal tissues (Ambs et al., 1998a). In human ovarian cancer, iNOS activity has been localized in tumour cells and not found in normal tissue (Thomsen et al., 1995). Other tumours that have demonstrated iNOS gene expression are brain, head and neck, esophagus, lung, prostate, bladder, pancreatic, and Kaposi's sarcoma (Cobbs et al., 1995; Rosbe et al., 1995; Wilson et al., 1998; Ambs et al., 1998a; Klotz et al., 1998; Hajri et al., 1998; Weninger et al., 1998; Swane et al., 1999).

In this study, we found that exist strong expression of iNOS in stroma and gland of HPB, in comparison with PCa. It Have been demonstrated that NO•- mediated up-regulation of VEGF. In the results is possible that NO• generated by iNOS in stroma may promote early new blood vessel formation by up-regulating VEGF and enhance ability of the tumour to grow and increases its invasiveness ability in gland. (Ambs et al., 1998a). Moreover, the accumulation of p53 in gland can result in down-regulation of iNOS expression by inhibition of iNOS promoter activity (Ambs et al., 1998b). On the other hand, the generation of chronic injury and irritation initiate the inflammatory response of stroma to gland (NOS-1). A subsequent respiratory burst an increase uptake of oxygen that leads to the release of reactive oxygen species (NO•, ONOO^- , N_2O_3 , NO_2 and NO_3) from leucocytes can damage surrounding cells and drive carcinogenesis by altering targets and pathways that are crucial to normal prostate homeostasis (Coussens & Werb, 2002; Fukumura et al., 2006).

COX-1 and COX-2 regulate a key step in prostanoid (i.e., tromboxanes and prostaglandins) synthesis. Prostaglandins regulate various pathophysiological processes such as inflammatory reaction, gastrointestinal cytoprotection and ulceration (Smith & Langenbach,

2001). COX-1 is the constitutive isoform and COX-2 is the inducible isoform. COX-1 is expressed in most tissues and plays a role in the production of prostaglandins that control normal physiological processes. COX-2 is undetectable in most normal tissues (except for the central nervous system, kidneys and seminal vesicles), but is induced by various inflammatory and mitogenic stimuli (growth factors, pro-inflammatory cytokines and tumor necrosis factor) and other regulatory factors (Peppelenbosch et al., 1993; Zhang et al., 1998, Chen et al., 2001; Dempke et al., 2001). Although the mechanism of COX-2 upregulation is not fully understood, it could result from activation of Ras and mitogen-activated protein kinase (MAPK) pathway. It has been recognized that Akt/PKB activity is implicated in Ras-induced expression of COX-2. COX-2 is regulated at transcriptional and post-transcriptional levels by proinflammatory agents. These pathways lead to the activation of regulatory factors that eventually bind the promoter region of the COX-2 gene. (Sheng et al., 1998, 2000).

In this study, we found that exist strong expression of COX-2 in stroma and gland of HPB, in comparison with PCa. There are conflicting data regarding whether COX-2 is increased in the epithelial, gland or the stromal component of tumors (Horsman et al., 2010). Liu et al were the first to describe tumorigenesis induced by COX-2 over-expression. In their study, the murine COX-2 gene was inserted downstream of a murine mammary tumor virus promoter. As a consequence, hyperplasia and carcinoma of the mammary gland were observed and associated with strong COX-2 expression in mammary gland epithelial cells with increase prostaglandin E2 levels. (Liu et al., 2001). The role of COX-2 in tumor promotion is more strongly supported by previous studies in colorectal tumor models describen by Oshima et al (Oshima et al., 1996). These findings have been confirmed analyzing many tumors including pancreas, skin, gastric, bladder, lung, head, and neck cancers, suggesting that COX-2, but not COX-1, may play a pivotal role in tumor formation and growth (Thun et al., 2002). COX-2-derived prostaglandins contribute to tumor growth by inducing angiogenesis that sustain tumor cell viability and growth. COX-2 is expressed within human tumor neovasculature as well as in neoplastic cells present in human colon, breast, prostate and lung cancer biopsy tissue. (Kerbel & Folkman, 2002). The proangiogenic effects of COX-2 are mediated primarily by three products of arachidonic metabolism: Tromboxane A2, Prostaglandins I2 and E2 and selective inhibition of COX-2 activity has been shown to suppress angiogenesis *in vitro* and *in vivo* (Tsujii et al., 1998; Masferrer et al., 2000; Uefuji et al., 2000). We suggested that COX-2 overexpression in stroma inhibit apoptosis and promote angiogenesis in prostate gland.

Our results suggest that iNOS and COX-2 play a key role in tumorigenesis and indicate that iNOS and COX-2-selective inhibitors could be a novel class of therapeutic agents for PCa.

5. Conclusions

We suggested that the $O_2^{\bullet-}/H_2O_2$ balance regulated by the over-expression of NOX, Cu/Zn-SOD, Mn-SOD and CAT is actively involved in tumor environment, cell proliferation, differentiation, tumor progression and angiogenesis of PCa. On the other hand, iNOS and COX-2 may promote blood vessel formation in gland from its over-expression in stroma by multiple mechanisms that involve reactive oxygen species, transcription factors, cytokines, growth factors and tumor necrosis factor.

Moreover, we suggested that the NOX, Cu/Zn-SOD, Mn-SOD, CAT, iNOS and/or COX-2 in combination with PSA, could be a molecular markers or prognostic indicators for the early diagnosis and post-treatment monitoring of PCa.

6. Future research

Our research group is determining the gene expression and activity of nitric oxide synthases isoforms (eNOS, nNOS and iNOS), Mn-SOD, Cu/Zn-SOD, Glutathione peroxidase, Glutathione reductase, Glutathione-S-transferase, Catalase and Cyclooxygenase-2 to integrate the effect of the regulation of the antioxidant system in the development of prostate cancer and recently in breast cancer.

Actually, we begin a new line of research where we studied the gene expression and polymorphisms of some components of the cytochrome P450 system as well as its association with the risk of developing prostate cancer and breast cancer. We found a protein over-expression of CYP2W1, 4F11 and 8A1, orphan cytochromes, in prostate cancer. We hope to find a molecular marker or prognostic indicator for prostate cancer and breast cancer.

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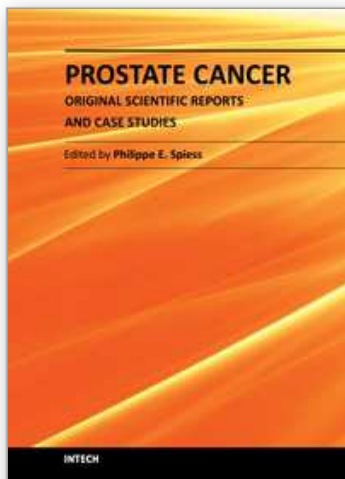
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This book encompasses three sections pertaining to the topics of cancer biology, diagnostic markers, and therapeutic novelties. It represents an essential resource for healthcare professionals and scientist dedicated to the field of prostate cancer research. This book is a celebration of the significant advances made within this field over the past decade, with the hopes that this is the stepping stone for the eradication of this potentially debilitating and/or fatal malignancy.

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