

TRAF-2/ASK-1/p38/NF- κ B pathways in normal prostate, benign prostatic hyperplasia and prostatic cancer

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This study was designed in order to analyze the expression of the transduction pathways TRAF-2/ASK-1/MEK-6/Pp38 and NF- κ B/p65, involved in the proliferation/apoptosis equilibrium in normal and pathological conditions. The study was carried out in 5 Normal Prostates (NP), 24 Benign Prostate Hyperplasia (BPH) and 19 Prostate Cancers (PC). Immunohistochemical and Western blotting analysis was performed. Western Blotting analysis revealed immunoexpression of TRAF-2, ASK-1, MEK-6 and Pp38 in BPH and PC. MEK-6 and NF- κ B/p65 were absent in NP. Immunohistochemical analysis showed significant weak optical density to ASK-1 in PC compared to NP and BPH. Optical densities to MEK-6 and NF- κ B/p65 were significantly intense in PC. p38 activation in prostate cancer could have apoptotic and proliferation role depending on the activation of its upstream and downstream component.

Key words: TRAF-2, ASK-1, p38, NF- κ B, apoptosis.

INTRODUCTION

Tumor Necrosis Factor (TNF) receptor associated factors (TRAFs) have emerged as the major signal transducers for the TNF receptor superfamily and the interleukin-1 receptor/Toll-like receptor superfamily (Wajant & Scheurich, 2001). TNF- α binding to its receptor TNFR-1 induces the activation of TRAF-2 (one of the six members of the TNF receptor associated factors) (Bradley & Pober, 2001). The signaling pathways activated by TRAF-2 results in different cell responses including survival, proliferation and apoptosis. TRAF-2 activated two major transcription factors: Activator Protein-1 (AP-1) and Nuclear Factor- κ B (NF- κ B) (Chung *et al.*, 2002; Wajant *et al.*, 2003; Hayden & Ghosh, 2008). Both transcription factors are activated through the activation of

JNK/p38 or I κ B kinases (Liu *et al.*, 2000). The TRAF-2-mediated NF- κ B activation promotes the proliferation by expression of genes involved in inflammatory and anti apoptotic responses (Bonizzi & Karin, 2004). Alternatively, TRAF-2 has also been shown to associate with Apoptosis Signal-regulating Kinase 1 (ASK1) and MEK-6 to induce p38 activation (Liu *et al.*, 2000). p38 is required for expression of TNF α and IL-1 inflammatory responses and most stimuli that activate p38 also induce expression of the p38 protein which regulates many transcription factors (including ELK-1, ATF-2, NF- κ B), cell cycle and apoptotic mediators (Zarubin & Han, 2005; Royuela *et al.*, 2008; Thornton & Rincon, 2009; Whyte *et al.*, 2009). Prostate cancer progression was characterized by activation of several signaling pathways, leading to the loss of proliferation and cell death balance (Ramsay & Leug, 2009; Culig & Pühr, 2011). These signaling are mainly induced by the expression of growth factors and pro-inflammatory cytokines (Ghosh *et al.*,

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2005; Bouraoui *et al.*, 2008). We have previously reported the expression of p38 transduction pathway triggered by IL-1 and its upstream (PAK-1/MEK-6) in human prostate tissue (Ricote *et al.*, 2006). In another work, we found that p38 is activated mediated TRAF-2/ASK-1 at MEK-4 step in normal human prostate (Ricote *et al.*, 2003). At present, and to our knowledge, no studies relating TRAF-2/ASK-1/MEK-6 to key members of p38 transduction pathway in prostatic tissue have been reported.

The aim of this study was to investigate the upstream (TRAF-2, ASK1, MEK-6) components of p38 pathways in conjunction with the transcription factor NF- κ B in normal prostate (NP), its profiles in benign prostatic hyperplasia (BPH) and prostatic carcinoma (PC) and its involvement in the control of proliferation/apoptosis equilibrium.

MATERIALS AND METHODS

Prostates were obtained from (a) transurethral resections from 24 men (aged from 55 to 85 years) diagnosed clinically and histopathologically as Benign Prostate Hyperplasia (BPH), (b) radical prostatectomy from 19 men (aged from 57 to 88 years) diagnosed with Prostate Cancer (PC) (Gleason scores 3 to 5) and (c) histologically Normal Prostates (NP) obtained at autopsy (8-10 hrs after death) from five men (aged from 20 to 38 years) without histories of reproductive, endocrine or related diseases. Each diagnosed sample was divided into two portions; one portion was immediately processed for the immunohistochemistry test, and the other portion was frozen and maintained at -80°C for Western blot analysis.

All pathological, clinical and personal data were anonymized and separated from any personal identifiers. All the procedures followed were examined and approved by the Department of Urology of Military Hospital of Tunis (HMPIT) and Hospital of La Rabta (Tunisia).

Antibodies

The primary antibodies used were mouse anti-human TRAF-2, p38 and NF- κ B (p-65), goat anti-human MEK-6 and ASK-1 (Santa Cruz Biotechnology, CA, USA), phospho-p38 (Pp38) rabbit anti-human (Cell Signaling Technology, Beverly MA, USA) and anti-chicken α -actin (Amersham, Buckinghamshire, UK).

For Western blot analysis, each prostate tissue was homogenized in the extraction buffer with addition of a cocktail of protease inhibitors and phosphatase

inhibitors. After centrifugation, aliquots of 10 μl of the homogenate were separated in SDS-polyacrylamide slab mini gels (15% gradient gels). Separated proteins were transferred to nitrocellulose membranes (0.2 μm), and were then blocked for 1 hr in TBS containing 1% donkey serum, and incubated overnight at room temperature with the primary antibodies at 1:50 (MEK-6), 1:100 (TRAF-2, ASK-1, p38), 1:200 (NF- κ B/p-65), 1:1000 (Pp38) and 1:10000 actin in TBS with 5% Bovine Serum Albumin (BSA). After extensive washing with TBS/Tween-20 (TBST), the membranes were incubated with swine anti-rabbit (Pp38), rabbit anti-mouse (TRAF-2, p38 and NF- κ B/p-65) and rabbit anti-goat (ASK-1 and MEK-6) biotinylated immunoglobulins (Dako, Barcelona, Spain) and mouse anti-chicken α -actin (Amersham, Madrid, Spain) for 1 hr at 1:4000 dilution in TBS with 5% BSA. Afterwards, the membranes were washed and incubated with the avidin-biotin-peroxidase complex (Vector Laboratories, Burlingame, CA, USA) at 1:10000 dilutions. After an intensive wash, the filters were developed with an enhanced chemilluminescence (ECL) kit (Amersham, Buckinghamshire, UK).

For immunohistochemistry analysis, tissues were fixed with 10% formaldehyde, dehydrated and embedded in paraffin. Sections (5 mm thick) were processed following the avidin-biotin-peroxidase complex (ABC) method. After deparaffinization, sections were hydrated, incubated for 30 min in 0.3% H_2O_2 diluted in methanol to reduce endogenous activity. To retrieve the antigen, the sections were incubated with 0.1 M citrate buffer (pH 6) for 2 min in a conventional pressure cooker. After incubation with TBS containing 3% donkey serum, the primary antibodies were applied at a dilution of 1:50 (TRAF-2, MEK-6, p38 and NF- κ B/p-65) and 1:25 (ASK-1 and Pp38) in TBS at 37°C overnight. Afterwards, the sections were washed twice and were incubated with secondary antibodies biotinylated immunoglobulin (Dako, Barcelona, Spain) at 1:500 dilutions in TBS for 1 hr. The sections were incubated with a standard streptavidin-biotin-complex (Vector Laboratories, Burlingame, CA, USA) at 1:500 and developed with 3,3'-diaminobenzidine (DAB), using the glucose oxidase-DAB-nickel intensification method. Immunohistochemical procedure specificity was checked using negative controls (sections incubated with preimmune serum or blocking peptides; Santa Cruz Biotechnology) and positive controls (human skin).

A comparative quantification (western blot and immunohistochemistry) of immunolabeling among

the different types of prostates was performed for each of the four antibodies. For each prostate, six histological sections were selected at random. In each section, the staining intensity (optic density) per unit surface area was measured with an automatic image analyzer (Motic Images Advanced version 3.2, Motic China Group Co., China) in 5 light microscopy fields per section, using the $\times 40$ objective. Delimitation of surface areas was carried out manually using the mouse of the image analyzer. For each positively immunostained section, one negative control section (the following in a series of consecutive sections) was also used, and the optic density of this control section was taken away from that of the stained section. From the average values obtained (by the automatic image analyzer) for each prostate, the mean values \pm SD for each prostatic type (NP, BPH and PC) were calculated. The same results were obtained by two different observers. The number of sections examined was determined by successive approaches to obtain the minimum number required to reach the lowest SD. The statistical significance between means of the different prostate group's samples was assessed by the Fisher exact test, the one-way ANOVA test and t test at $p < 0.05$ by GraphPad Prism 3.0 (GraphPad Software, Inc. San Diego, USA).

RESULTS

Western blot analysis

For each antibody used, a single band – at their corresponding molecular weight – was found in the three groups to TRAF-2 (52 kDa), ASK-1 (165 kDa) and Pp38 (38 kDa). MEK-6 (80-90 kDa) and NF- κ B/p65 (65 kDa) were not detected only in NP, while p38 (38 kDa) was negative only in PC (Fig. 1).

Comparison of optical density revealed significant differences among the three prostate groups (Table 1). Average optical densities to MEK-6, Pp38 and NF- κ B/p65 increased with malignancy. Average optical density to ASK-1 was higher in BPH than in PC ($p < 0.05$). No significant differences in the average optical densities to TRAF-2 were found in NP and PC ($p > 0.05$).

Immunohistochemistry analysis

No immunoreaction was observed in the negative controls incubated with pre-immune serum, or the antibodies pre-absorbed with an excess of purified antigens. Skin samples showed immunoreactions for all antibodies used as positive controls. No significant histological or quantitative immunohistochemical differences between the two subgroups of normal pro-

FIG. 1. Western blot analysis of TRAF-2, ASK-1, MEK-6, p38, Pp38, NF- κ B/p65 and α -actin after 15% polyacrylamide gel electrophoresis. NP: normal prostate. BPH: benign prostate hyperplasia. PC: prostate carcinoma. The lanes showing a band correspond to a positively stained prostate gland from each group. No immunoreactions were found to NF- κ B/p65 and MEK-6 in normal prostate.

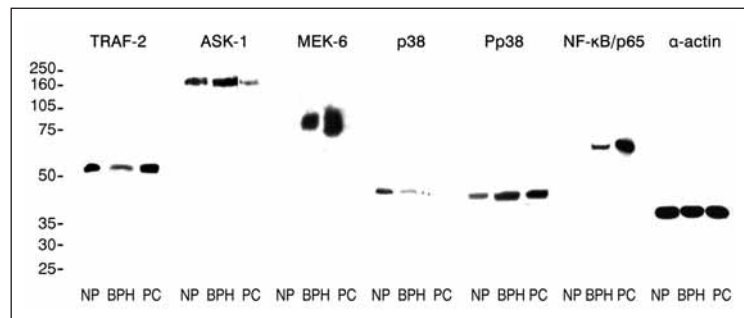


TABLE 1. Comparison of immunostaining intensities (measured as average optical density \pm SD) in Western blot analysis in Normal Prostate (NP), Benign Prostatic Hyperplasia (BPH) and Prostate Cancer (PC). Average optical densities were only evaluated in patients showing positive immunoreactions. For each antibody (column) values with different superscript letters differ significantly ($p < 0.05$)

	TRAF-2	ASK-1	MEK-6	p38	Pp38	NF- κ B/p65
NP	32.56 \pm 2.14 ^a	21.47 \pm 2.4 ^a	–	15.87 \pm 1.9 ^a	18.52 \pm 3.3 ^a	–
BPH	19.03 \pm 3.41 ^b	38.48 \pm 2.4 ^b	34.67 \pm 4.2 ^a	7.48 \pm 3.2 ^b	28.67 \pm 2.6 ^b	11.67 \pm 32.3 ^a
PC	31.56 \pm 2.8 ^a	8.27 \pm 2.2 ^c	49.76 \pm 2.1 ^b	–	34.74 \pm 3.1 ^c	58.90 \pm 3.6 ^b

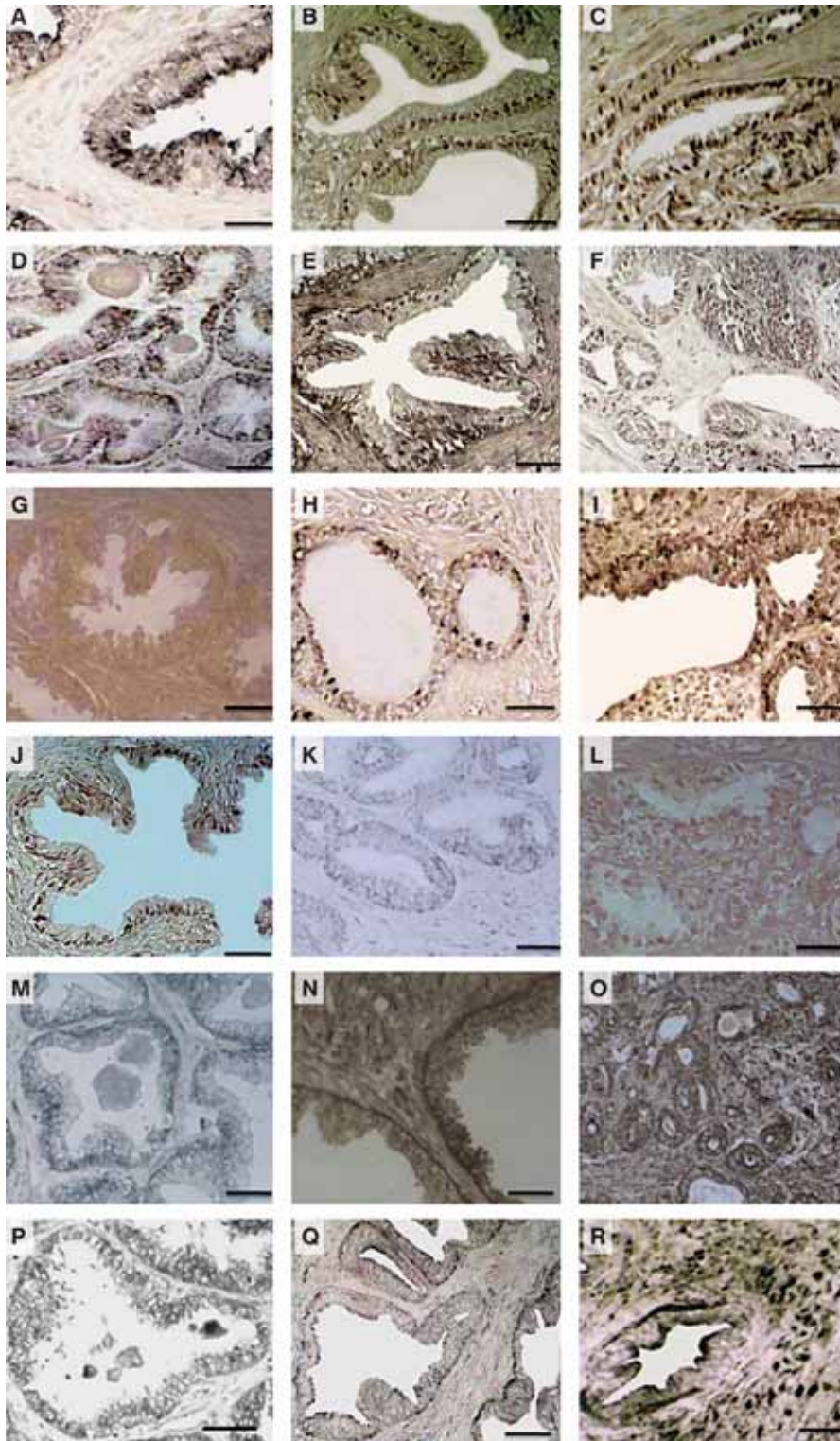


FIG. 2. TRAF-2 was present in epithelial cells in NP (A), BPH (B) and PC (C). ASK-1 immunostaining appeared in the epithelial cells of NP (D), BPH (E) and PC (F); weak immunoreactions were observed in PC (F). MEK-6 was negative in NP (G), but the cytoplasm of the epithelial cells presented a positive immunoreaction in BPH (H) and PC (I). p38 presented an immunoreaction in epithelial cells of NP (J) and BPH (K); weak immunoreactions were detected in PC (L). Pp38 presented immunoreaction in the nuclei and the cytoplasm of epithelial cells of NP (M), BPH (N) and PC (O). NF- κ B was negative in NP (P), but immunostaining appeared in the cytoplasm of epithelial cells of BPH (Q); however, in PC samples (R), immunoreactions were also localized in the nuclei of epithelial cells. Scale bars: 30 μ m (A, M, N, P, R), 25 μ m (G, H, I, K, L), 20 μ m (B-D, E, J) and 40 μ m (F, O, Q).

TABLE 2. Percentage of patients showing positive immunohistochemical reactions to TRAF-2, ASK-1, MEK-6, Pp38, p38 and NF- κ B (p65) in Normal Prostate (NP, n = 5), Benign Prostatic Hyperplasia (BPH, n = 24) and Prostate Cancer (PC, n = 19). Average optical densities (O.D.) were only evaluated in patients showing positive immunoreactions. Values denoted by different superscripts are significantly different from each other. Those values sharing the same superscript are not statistically different ($p > 0.05$). Statistical analysis refers to each antibody separately

<i>Patients</i>	TRAF-2		ASK-1		MEK-6		p38		Pp38		NF- κ B/p65	
	%	OD	%	OD	%	OD	%	OD	%	OD	%	OD
NP	100	40.59 ± 1.99 ^a	100	18.27 ± 1.84 ^a	0	1.05 ± 0.54 ^a	100	14.66 ± 2.22 ^a	40.00	32.58 ± 1.59 ^a	0	0.08 ± 0.1 ^a
BPH	58.30	31.41 ± 1.6 ^b	75	29.01 ± 1.94 ^b	54.16	31.01 ± 1.75 ^b	25	5.87 ± 0.99 ^b	58.30	39.60 ± 2.8 ^b	38.09	18.46 ± 2.04 ^b
PC	52.60	41.44 ± 2.05 ^a	100	9.26 ± 1.95 ^c	52.60	50.74 ± 1.89 ^c	5.2	0.9 ± 0.04 ^c	78.94	51.68 ± 2.75 ^c	41.17	24.18 ± 0.67 ^{c*} 27.54 ± 1.60 [#]

* Cytoplasmic immunoreaction

Nuclear immunoreaction

states (biopsies and autopsies) were observed.

Normal prostates (NP): For the protein kinase MEK-6 and the transcription factor NF- κ B/p65, no positive immunoreactions were observed in NP (Fig. 2G). However, in all normal prostates, TRAF-2, ASK-1 and p38 were localized to cytoplasm epithelial cells (Fig. 2A, 2D, 2J), whereas Pp38 was present in the nucleus of epithelial cells in 40% of NP specimens (Table 2 and Fig. 2M).

BPH and PC patients: In all proteins studied, immunostaining were positive especially for MEK-6 and the subunit NF- κ B/p65 (Table 2). In the 58.3% of BPH and the 52.6% of PC samples, TRAF-2 appeared in the cytoplasm and the nucleus of epithelial cells (Fig. 2B, 2C). Optical densities revealed a weak immunostaining to TRAF-2 in BPH compared to NP and PC, but no statistical differences were observed between them (Table 2). Positive immunoreactions to ASK-1 appeared in 75% of BPH samples localized in the cytoplasm epithelial cell with a stronger immunoreaction compared to PC (Fig. 2E, 2F). Indeed, optical density to ASK-1 was significantly higher in BPH than in PC samples ($p < 0.05$) (Table 2).

The percentage of positive samples to MEK-6 was similar in BPH (54.14%) and PC (52.16%) patients observed in the cytoplasm of epithelial cells (Fig. 2H, 2I). The highest optical density was found in PC patients (Table 2). No immunoreactions to p38 were observed in PC samples. In 25% of BPH samples, immunoreactions were observed in the cytoplasm of epithelial cells but the intensities were lower than those observed in NP (Fig. 2J, 2K, 2L). Pp38 was found in the nucleus and the cytoplasm of epithelial cells in 58.3% of BPH and 78.9% of PC samples (Fig. 2N, 2O). A higher optical density was detected in PC samples (Table 2).

Positive immunoreactions to NF- κ B/p65 in the 38% of BPH patients were detected in the cytoplasm of epithelial cells whereas in the 41% of PC samples were observed in both cytoplasm and nucleus of epithelial cells (Fig. 2Q, 2R). Optical density was higher in PC than in BPH patients (Table 2).

DISCUSSION

There is strong evidence that inflammation and prostate cancer were associated with imbalance between proliferation and apoptosis. TRAF-2 contributes significantly to the maintenance of equilibrium in normal tissues through AP-1 and NF- κ B activated by TNF- α pathways (Wajant *et al.*, 2003). In normal pro-

states, immunoexpressions to TRAF-2 were associated to immunoexpressions of ASK-1, Pp38 and p38, but no immunoreactions to MEK-6 and NF- κ B/p65 were found. Like to MEK-6, MEK-4 kinase is required for p38 activation (Korchnak *et al.*, 2009). The immunoexpressions of MEK-4 and p38 in normal prostates have been demonstrated as the inhibition of p38 activation via PAK-1 and MEK-6, suggesting that p38 activation by TRAF-2 is triggered by TNF- α via MEK-4 cascades (Ricote *et al.*, 2006). In normal prostate samples, TRAF-2 is immunoexpressed, but no NF- κ B, suggesting that the ability of TRAF-2 to activate p38 and NF- κ B raises the question regarding the specific functions of TRAF-2. Therefore, signaling pathways mediated by TRAF-2 may lead to preferential activation of specific NF- κ B and p38 components. In addition, to trigger NF- κ B, TRAF-2 must activate the I κ B Kinases (IKK) complex (Devin *et al.*, 2000; Lee *et al.*, 2004). In previous papers, we have shown the expression of pro inflammatory cytokines (TNF α , IL-6, but not IL-1 α) in normal prostates, strong activators of IKK complex and consequently NF- κ B (Gasparian *et al.*, 2002; Bouraoui *et al.*, 2008). Based on these finding, we suggest that prostate epithelial cells are insufficient to induce NF- κ B activation.

In BPH samples, all studied factors were expressed with a strong immunoexpression of ASK-1 in conjunction with Pp38. This data suggests that active stimulation of cell death mediated TRAF-2/ASK-1/MEK-6/p38 occurs. ASK-1 is known to play crucial role in apoptosis as well as stress or pro inflammatory cytokines (Hattori *et al.*, 2009). In addition, activation of TNF- α /TRAF-2 signal pathway mediated by ASK-1 is due to the excessive production of oxidative stress by cells under inflammatory and stress conditions (Hayakawa *et al.*, 2006).

Using the same samples as those in this study, we observed the expression of TNF- α and its receptor TNFR-1 in BPH samples (Bouraoui *et al.*, 2008), suggesting that TRAF-2/ASK-1/MEK-6/p38 apoptotic pathway is activated in an attempt to limit the inflammatory cell damages in cooperation with other apoptotic signaling (Ricote *et al.*, 2006). In oxidative stress, TRAF-2 and ASK-1 form a high-molecular-mass complex that, in turn, induces prolonged apoptosis by JNK (Noguchi *et al.*, 2005). Immunoreaction to NF- κ B/p65 was detected in the cytoplasm of epithelial cells. It is well known that I κ B proteins retain NF- κ B in the cytoplasm in non-pathological conditions (Li & Verma, 2002). Recent studies have indicated the increase of pI κ B (Gloire *et al.*, 2006; Nuñez

et al., 2008) and H₂O₂ is responsible for inducing IKK β . However, the increase of pIkB, perhaps, is insufficient to transport the subunit NF- κ B/p65 to the nucleus.

In PC samples, weak immunoreaction to ASK-1 was found; on the contrary, intense expressions for MEK-6, Pp38 and NF- κ B/p65 were detected. These findings suggested that the apoptotic pathway TRAF-2/ASK-1/MEK-6/p38 seems to be inactive, since immunoreactions to ASK-1 decreased. In this microenvironment, tumor cells displace the proliferation/apoptosis equilibrium toward proliferation by inhibition of many apoptotic events. Therefore, ASK-1 may be blockade by anti-apoptotic protein such as p21, GST family member and some phosphatases which inactivate ASK-1 (Dolado et al., 2007; Kennedy et al., 2007). In addition, NF- κ B may inhibit ASK-1 activation (Mochida et al., 2000). Importantly, recent studies have shown that both TNF- α and oxidative stresses induced TRAF-2 phosphorylation dependent recruitment of IKK to the TRAF-2/ASK1/RIP1 complex in all cell types, leading to activate NF- κ B and cell death inhibition by suppressing prolonged activation of JNK, AP-1 (Ricote et al., 2003; Zhang et al., 2011). Since p38 and JNK have common downstream components pathways mediated by TRAF-2, we hypothesized that in our prostate samples, NF- κ B activation could be mediated by TRAF-2 involved to cell proliferation. Translocation of NF- κ B/p65 to the nucleus in PC could be stimulated by other signaling pathways triggered by pro inflammatory cytokines such as IL-6 (Paule et al., 2007; Rodríguez-Berriguette et al., 2010). However, activation of NF- κ B/p65 could be inhibited by IL-1 β in LNCaP cell lines (Bouraoui et al., 2012).

p38 activation induces apoptosis, but it is also required for cell survival and differentiation (Kyriakis & Avruch, 2001). In our prostate cancer tissues, we found high activation of p38 phosphorylated (Pp38), MEK-6 and NF- κ B/p65 (proliferation signal) associated to the weak intensities of ASK-1 (pro-apoptotic signal).

These findings suggested that p38 is associated with cancer progression (Evan & Vousden, 2001; Kennedy & Davis, 2003) by activation transcription factors related with proliferation including ATF-2, ELK-1 (Xiao et al., 2002; Recio & Merlino, 2003) and NF- κ B (Madrid et al., 2001; Kato et al., 2003; Vermeulen et al., 2003). Triggering of apoptotic or proliferation p38-mediated response seems to depend on the stimuli, cellular microenvironment and the p38

isoform involved (Ricote et al., 2006; Feng et al., 2009). Therefore, and in spite of displaying prostatic functions, p38 transduction pathway may constitute a target for prostate cancer treatment by androgen dependence contribution and metastatic phenotype acquisition.

Like to TRAF-2, TRAF-6 was expressed in prostate tissues (data not shown) and seems to be a determinant of cell balance (proliferation/apoptosis) by activation of NF- κ B and p38 upon pro-inflammatory cytokines (Korchnak et al., 2009). It thus appears that the ratio TRAF-2/TRAF-6 might be important mediators triggering cell life or death. Future studies should be focused on identify different mechanisms involved in TRAFs recruitment and lead to preferential activation of specific apoptotic and proliferation components related to prostate specific proteins activated by pro-inflammatory cytokines. Those will be important in developing new strategies to prevent excessive tumor cell proliferation pathways.

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