

RESEARCH ARTICLE

Relationship between IL-6/ERK and NF- κ B: a study in normal and pathological human prostate gland

Gonzalo Rodríguez-Berriguete¹, Angela Prieto¹, Benito Fraile¹, Yosra Bouraoui⁴,
Fermín R. de Bethencourt³, Pilar Martínez-Onsurbe², Gabriel Olmedilla², Ricardo Paniagua¹,
Mar Royuela¹

¹ Department of Cell Biology and Genetics. University of Alcalá, Madrid, Spain

² Department of Pathology, Príncipe de Asturias Hospital, Madrid, Spain

³ Department of Urology, "La Paz" Hospital, Madrid, Spain

⁴ Unité d'Immunologie Microbiologie à la Faculté des Science de Bizerte, Tunisie

Correspondence: M. Royuela, Department of Cell Biology and Genetics. University of Alcalá, 28871 Alcalá de Henares, Madrid, Spain
<mar.royuela@uah.es>

Accepted for publication July 30, 2010

ABSTRACT. Background. There is growing evidence that inflammation is a causal factor in cancer, where pro-inflammatory cytokines such as IL-6, IL-1 or TNF- α could induce cellular proliferation by activation of NF- κ B. This study focuses on the IL-6/ERK transduction pathway, its relationship with NF- κ B, and the consequences of dysregulation in the development of prostate pathologies such as benign prostate hyperplasia (BPH), prostate intraepithelial neoplasia (PIN) and prostate cancer (PC). **Methods.** Immunohistochemical and Western blot analyses for IL-6, gp-130, Raf-1, MEK-1, ERK-1, p-MEK, ERK-2, p-ERK, NF- κ B/p-50 and NF- κ B/p-65 were carried out in 20 samples of normal prostate glands, 35 samples of BPH, 27 samples with a diagnosis of PIN (low-grade PIN or high-grade PIN), and 95 samples of PC (23 with low, 51 with medium and 21 with high Gleason scores). **Results.** Immunoreaction to IL-6, gp-130, ERK-1, ERK-2, p-ERK and NF- κ B/p50 was found in the cytoplasm of epithelial cells in normal prostate samples; p-MEK was found in the nucleus of epithelial cells; but not expression to Raf-1, MEK-1 and NF- κ B/p65. In BPH, all of these proteins were immunoexpressed, while there was increased immunoexpression of IL-6, gp-130, p-MEK, ERK-1, ERK-2 and NF- κ B/p50 (cytoplasm). In PC, immunoexpression of IL-6 and gp-130 were similar to that found in BPH; while immunoexpression of Raf-1, MEK-1, p-MEK, ERK-1, ERK-2, p-ERK, NF- κ B/p50 (nucleus and cytoplasm), and NF- κ B/p65 (nucleus and cytoplasm) was higher than in BPH. **Conclusion.** Translocation of NF- κ B to the nucleus in PC and high-grade PIN could be stimulated by the IL-6/ERK transduction pathway, but might also be stimulated by other transduction pathways, such as TNF- α /NIK, TNF/p38, IL-1/NIK or IL-1/p38. Activation of NF- κ B in PC could regulate IL-6 expression. These transduction pathways are also related to activation of other transcription factors such as Elk-1, ATF-2 or c-myc (also involved in cell proliferation and survival). PC is a heterogeneous disease, where multiple transduction pathways might alter the apoptosis/proliferation balance. Significant attention should be give to the combination of novel agents directed towards inactivation of pro-inflammatory cytokines than can disrupt tumour cell growth.

Keywords: IL-6, MEK-1, Raf-1, ERK, NF- κ B, prostate cancer

Inflammation has been suggested to be a causal factor in several human tumors, including prostate cancer. This process causes cell and genome damages, promotes cellular replacement, and creates a cytokine-rich tissue microenvironment that can enhance cell replication, angiogenesis and tissue repair [1, 2]. The role of pro-inflammatory cytokines, such as IL-6, IL-1 and TNF- α , in triggering the activation of NF- κ B, has been well established in cancer [3].

IL-6 is able to transduce intracellular signaling through a receptor complex called IL-6R, which is composed of two subunits: gp80 (IL-6R α or sIL-6R) and gp130 (IL-6R β), which is a common receptor complex component for all IL-6 family members. While gp80 directly binds IL-6,

gp130 is responsible for signal transduction. The binding of IL-6 to IL-6R α induces dimerization of gp130 [4], and subsequently the activation of constitutively-associated gp130 Jak proteins. Jak proteins can simultaneously trigger functionally distinct and even contradictory signaling pathways. One of them leads to the recruitment, at the receptor complex of SHP2, Sos and Grb2, which in turn activates Ras by stimulating the exchange of GDP bound to Ras for GTP. Then, Ras initiates a MAPK cascade by sequential phosphorylation of Raf-1, MEK1/2 and ERK1/2, in a process that culminates in modulation of gene transcription through the activation of several transcription factors such as c-Myc, ATF-2, Elk-1 [5] or NF- κ B [6]. MEK1/2 may also be phosphorylated by

TRAF-2 or TRAF-6. Moreover, ERK may also induce the phosphorylation of apoptotic regulatory molecules including Bcl-2 family members (*e.g.* Bad, Bim and controversially Bcl-2) and caspase 9 [7]. There is evidence suggesting a protective effect in cells bestowed by NF- κ B activation *via* ERK [8, 9]. This transcription factor, in a basal state, is retained in the cytoplasm by binding to specific inhibitors, the inhibitors of NF- κ B (I κ Bs). Upon cell stimulation, I κ Bs are degraded, and consequently NF- κ B is translocated into the nucleus [10], where it promotes the expression of several anti-apoptotic genes such as inhibitors of apoptosis proteins (IAPs) [11] and Bcl-2 family members [12]. At the same time, NF- κ B may promote cell growth and proliferation in prostate cancer cells by regulating expression of genes such as IL-6 [13]. Characterization of the IL-6 promoter has revealed a complex control region with binding motifs for NF- κ B [14, 15].

The mammalian ERK pathway is implicated in several physiological cell processes, including cell proliferation, differentiation and survival [6]. Some components of the Raf/MEK/ERK pathway are activated in solid tumors and hematological malignances [7, 16, 17]. The implication of the Raf/MEK/ERK pathway in drug resistance in some cancer cells such as breast or hematopoietic cancer cells has been demonstrated [7]. Increased expression of the Raf pathway has been associated with advanced prostate cancer, hormonal independence, metastasis and a poor prognosis [18, 19]. Moreover, prostate cancer cell lines isolated from advanced cancer patients (LNCaP, PC3 and DU145) expressed low levels of active Raf kinase inhibitors [7, 20].

The activation of IL-6/ERK pathway *in vivo* has not yet been sufficiently addressed. We have previously reported the immunohistochemical expression and localization of ERK [21] and IL-6 [22] in normal prostate, benign prostatic hyperplasia (BPH) and prostatic carcinoma (PC); but no information has been reported regarding PIN patients. At present, and to our knowledge, no immunohistochemical studies on Raf-1 and MEK-1 in prostatic tissue have been reported, or studies relating them to key members of this transduction pathway. The aim of this study was to investigate the upstream (Raf-1 and MEK-1) and downstream (NF- κ B) components of the ERK transduction pathway in normal human prostate, their modifications in BPH, PIN and PC, and their possible involvement in proliferation and survival *via* IL-6 stimulation.

METHODS

Prostate glands were obtained from: (a) transurethral resections from 35 men (aged from 53 to 88 years) with the clinical and histopathological diagnosis of BPH; (b) autopsy or biopsy from 27 men (aged from 20 to 59 years), with a diagnosis of low-grade PIN (12 men) or high-grade PIN (15 men); (c) radical prostatectomies from 95 men (aged from 54 to 69 years) with PC of low (Gleason scores < 6, 23 men), medium (Gleason score 7, 51 men) and high (Gleason scores 8-10, 21 men) Gleason scores, with and without metastases or lymph node infiltration at the time of surgery and

(d) histologically normal prostates (NP) obtained at autopsy (8-10 hours after death) from 20 men (aged from 20 to 38 years) with no particular medical history or reproductive, endocrine or related diseases. Each sample was divided into two portions; one portion was immediately processed for immunohistochemistry, while the other was frozen in liquid nitrogen and maintained at - 80°C for Western blotting analysis. In this second portion, cryostat sections were stained with toluidine blue to confirm the histopathological diagnosis. All pathological, clinical or personal data were anonymized and separated from any personal identifiers. This study was performed with the consent of the patients, or their family in autopsy cases. All procedures followed were examined and approved by the University of Alcalá and the Principe de Asturias Hospital Ethics Committees (reference number SAF2007-61928), and were in accordance with the ethical standards of the Committee for Human Experimentation, with the Helsinki Declaration of 1975 (as revised in Tokyo 2004) and the Committee on Publication Ethics (COPE) guidelines.

The primary antibodies used were: rabbit anti-human IL-6, gp-130, p-MEK, ERK-1 and NF- κ B (p-50); mouse anti-human MEK-1, Raf-1, ERK-2, p-ERK and NF- κ B (p-65) (Santa Cruz Biotechnology, CA, USA); and chicken anti α -actin (Amersham, Buckinghamshire, UK). For Western blot analysis, we selected small portions after the study of several cryostat sections of unfixed tissues in order to make a selection of normal and pathological regions. Tissues were isolated and homogenized in the extraction buffer (0.005M Tris-HCl, pH 8) with the addition of a cocktail of protease inhibitors (10 mM iodoacetamide, 100 mM phenylmethyl sulphonyl fluoride, 0.01 mg/mL of soybean trypsin inhibitor and 1 μ L/mL of leupeptin) and phosphate inhibitors (10 mM sodium fluoride and 1 mM sodium orthovanadate) in the presence of 0.5% Triton X-100. Homogenates were centrifuged for 10 min at 10,000 rpm. The total protein concentrations of the supernatants were determined using the Bradford method. Supernatants were mixed with an equivalent volume of SDS loading buffer (10% SDS in Tris/HCl pH 8 containing 50% glycerol, 0.1 mM 2-beta-mercaptoethanol and 0.1% bromophenol blue). Then, the mixture was denatured for 5 min at 100°C, and aliquots of 10 μ L homogenate were separated on SDS-polyacrylamide slab minigels (15% gels). Separated proteins were transferred in the transfer buffer (25 mM Tris-HCl, 192 mM glycine, 0.1% SDS and 20% methanol). Nitrocellulose membranes (0.2 μ m) were blocked for 1 h with 1% donkey serum in TBS, and incubated overnight at room temperature with the primary antibodies at 1:200 [IL-6, MEK-1, p-MEK, Raf-1, ERK-2, ERK-1, p-ERK, NF- κ B (p-65) and NF- κ B (p-50)], 1:1000 (gp-130) and 1:10,000 chicken anti- α -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 [IL-6, gp-130, ERK-1 and NF- κ B (p-50)] and rabbit anti-mouse [MEK-1, Raf-1, ERK-2 and NF- κ B (p-65)] biotinylated immunoglobulins (Dako, Barcelona, Spain) for 1 h at 1:2500 dilution in TBS with 5% BSA. Furthermore, mouse anti-chicken α -actin (Amersham, Madrid, Spain) was also used at the same dilution to examine the

relative expression of the other proteins. After this incubation, membranes were washed and incubated with the avidin-biotin-peroxidase complex (Vector Laboratories, Burlingame, CA, USA) at 1:1,000 dilutions. After an intensive wash, the filters were developed with an enhanced chemiluminescence (ECL) kit, following the procedure described by the manufacturer (Amersham, Buckinghamshire, UK).

For light microscopy immunohistochemical study, tissues were fixed for 24 h at room temperature in 0.1 M phosphate-buffered 10% formaldehyde, dehydrated and embedded in paraffin. Sections (5- μ m thick) were processed following the avidin-biotin-peroxidase complex (ABC) method. Following deparaffinization, sections were hydrated, incubated for 30 min in 3% H₂O₂ diluted in methanol to reduce endogenous activity. To retrieve the antigen, sections were incubated with 0.1 M citrate buffer (pH 6) for 5 min in a conventional pressure cooker. After rinsing in TBS buffer, the slides were incubated with normal donkey serum at 10% in TBS for 30 min to prevent non-specific binding of the first antibody. Thereafter, the primary antibodies were applied at a dilution of 1:50 [IL-6, MEK-1, p-MEK, Raf-1, ERK-2, ERK-1, NF- κ B (p-65) and NF- κ B (p-50)], 1:100 (p-ERK) and 1:200 (gp-130) in TBS at 37°C, overnight. Afterwards, the sections were washed twice in TBS and then incubated with swine anti-rabbit [IL-6, gp-130, p-MEK, ERK-1 and NF- κ B (p-50)], rabbit anti-mouse [MEK-1, Raf-1, ERK-2 p-ERK, and NF- κ B (p-65)] immunoglobulin (Dako) at 1:500 dilutions. After 1 h of incubation with the secondary antibody, the sections were incubated with a standard streptavidin-biotin-complex (Vector) and developed with 3,3'-diaminobenzidine (DAB), using the glucose oxidase-DAB-nickel intensification method.

Immunochemical procedure specificity was checked using negative and positive controls. For negative controls, tissues of each type (normal, BPH and PC) were incubated with blocking peptides (Sta. Cruz Biotechnology) or pre-immune serum at the same immunoglobulin concentration used for each antibody. As positive controls, homogenates (for Western Blot) and histological sections (for immunohistochemistry) of human skin or thymus were incubated with the same antibodies.

A comparative histological quantification of immunolabeling among the different types of prostate gland samples was performed for each antibody. For each prostate gland, six histological sections were selected at random. In each section, the staining intensity (optical density) per unit surface area was measured with an automatic image analyzer (Motic Images Advanced version 3.2, Motic China Group Co., China) in five light microscopic fields per section, using the X40 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 optical density of this control section was subtracted from that of the stained section. From the average values obtained (by the automatic image analyzer) for each prostate, the means \pm SD for each prostate type (NP, BPH, PIN and PC) were calculated. The results were corroborated by two different observers. The statistical significance between means of the different prostate group samples was assessed by the one-way ANOVA test at $p \leq 0.05$, by multiple pairwise comparisons (GraphPad PRISMA 3.0 computer program).

To determine whether the source of material (surgery or autopsy) might be responsible for changes in the immunohistochemical pattern, five prostatic biopsies (taken because of the suspicion of prostatic disease: their histological examination revealed a normal pattern) were processed for immunohistochemistry. The results of the quantitative immunohistochemical study in these biopsies were compared with those of autopsied prostate glands.

RESULTS

Western blot analysis

Western blot analysis showed a single band for all the antibodies studied at the corresponding molecular weight in BPH and PC. In normal prostates (NP), MEK-1, Raf-1, NF- κ B/p65 was not detected, while immunoreactions for the other antibodies were found at the corresponding molecular weight (figures 1, 2).

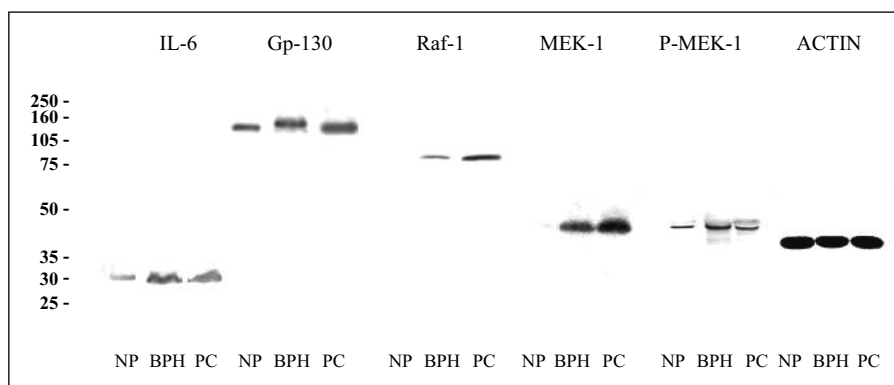
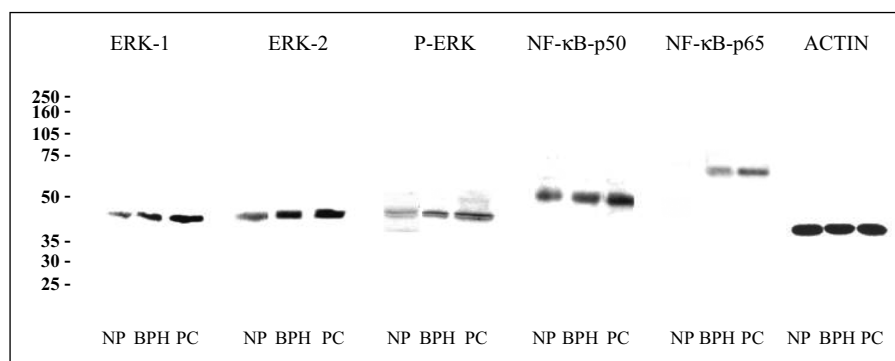


Figure 1

Western blot analysis of IL-6, gp-130, MEK-1, p-MEK, Raf-1 and α -actin after 15% polyacrylamide gel electrophoresis. NP: normal prostate. BPH: benign prostatic hyperplasia. PC: prostate carcinoma. The lanes showing a band correspond to a positively-stained prostate gland from each group. No immunoreaction was found to Raf-1 and MEK-1 in normal prostate.

**Figure 2**

Western blot analysis of ERK-1, ERK-2, p-ERK, NF- κ B (p50), NF- κ B (p65) and α -actin after 15% polyacrylamide gel electrophoresis. NP: normal prostate. BPH: benign prostatic hyperplasia. PC: prostate carcinoma. The lanes showing a band correspond to a positively stained prostate gland from each group. No immunoreaction was found to NF- κ B (p65) in normal prostate.

Immunohistochemistry

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 (Raf-1, MEK-1, p-MEK, ERK-1, ERK-2, p-ERK, NF- κ B) or thymus (IL-6 and gp-130) samples showed immunoreactions for all antibodies used as positive controls.

No significant histological or quantitative immunohistochemical differences between the two subgroups of normal prostates (biopsies and autopsies) were observed.

IL-6 and gp-130

IL-6 was observed in all normal prostates (*figure 3A*) and the low grade PIN of the epithelial cells, although with low intensity (*table 1*). However, in 80% of BPH specimens, IL-6 was present in the epithelial compartment (*figure 3B*), with a stronger immunoreaction compared to normal or low grade PIN prostates (*table 1*). In prostate carcinoma (*figure 3C*) and high grade PIN, the IL-6 immunoreaction was localized to the epithelial compartment (*table 1*). The highest optical density was found in BPH, PC and high-grade PIN, but no statistical differences were observed between them (*table 1*).

Positive immunostaining to gp-130 was observed in epithelial cells in all normal (*figure 3D*) and low grade PIN prostate glands; and in 57.14% of patients with BPH (*table 1*). In PC (more than 80% of patients) and high grade PIN (80% of patients), gp130 was also immunolocalized to epithelial cells (*table 1*). Optical density to gp-130 was similar in BPH, high grade PIN and PC, with low (*figure 3E*), medium and high Gleason score (*table 1*; *figure 3F*).

Raf-1

No immunoreaction to Raf-1 was found in normal prostate and low grade PIN (*figure 3G*; *table 1*). A positive immunoreaction was observed in the cytoplasm of epithelial cells in BPH (37.14% of patients) (*figure 3H*), high grade PIN (46.6%) and PC (more than 42%) (*figure 3I*; *table 1*). The highest optical density was found in PC and increased as a function of the Gleason score. No differences were observed between PIN (high grade) and a low Gleason score.

MEK-1

Immunoreaction to MEK-1 was absent in normal prostate glands and low grade PIN (*table 1*). A cytoplasmic immunoreaction in epithelial cells was observed in 62.85% of BPH (*figure 3J*), in 73.3% of PIN (high grade) (*figure 3K*), and in more than 50% of PC patients (*figure 3L*; *table 1*). Optical density was higher in PC samples and PIN (high grade). At the same time in PC groups, optical density increased with the Gleason score. p-MEK always showed immunoreaction in the nuclei in 40% normal prostate glands (*figure 3LL*; *table 1*), 41.6 of low grade PIN, 68.57% of BPH patients, 73.33% of high grade PIN (*figure 3M*), 71.42% of cancer patients with a low Gleason score (*figure 3N*), 78.43% of cancer patients with a medium Gleason score, and 80.95% cancer patients with a high Gleason score. Optical density was higher in PC samples than in normal prostate glands and BPH. Optical density was higher in BPH samples and high grade cancer than in the other groups. A lower optical density was found in normal prostate and low-grade PIN (*table 1*).

ERK

Immunostaining to ERK-1 appeared in the cytoplasm of epithelial cells in 80% of normal prostate glands, patients with BPH (*figure 4A*; *table 2*) and patients with low-grade PIN: 86.6% of patients with high grade PIN and more than 90% PC samples were positive (*figure 4B,C*; *table 2*). Optical density was higher in BPH samples than in normal prostate glands and low-grade PIN. The highest optical density was found in PC and high grade PIN. No differences were observed between high-grade PIN and low Gleason scores. In PC, optical densities were increased in line with Gleason scores (*table 2*).

Immunoreaction to ERK-2 was present in epithelial cells in 100% of normal prostate glands (*figure 4D*; *table 2*), 71.42% of BPH patients, 100% of low-grade PIN samples, 86.6% of high-grade PIN (*figure 4E*), 80.95% of PC (*figure 4F*) with a low Gleason score, 82.35% of PC with a medium Gleason score, and 76.19% of PC with a high Gleason score. A lower optical density was found in normal prostate gland and low- grade PIN; the highest in PC and high grade PIN, but no differences between Gleason groups and high-grade PIN were found (*table 2*).

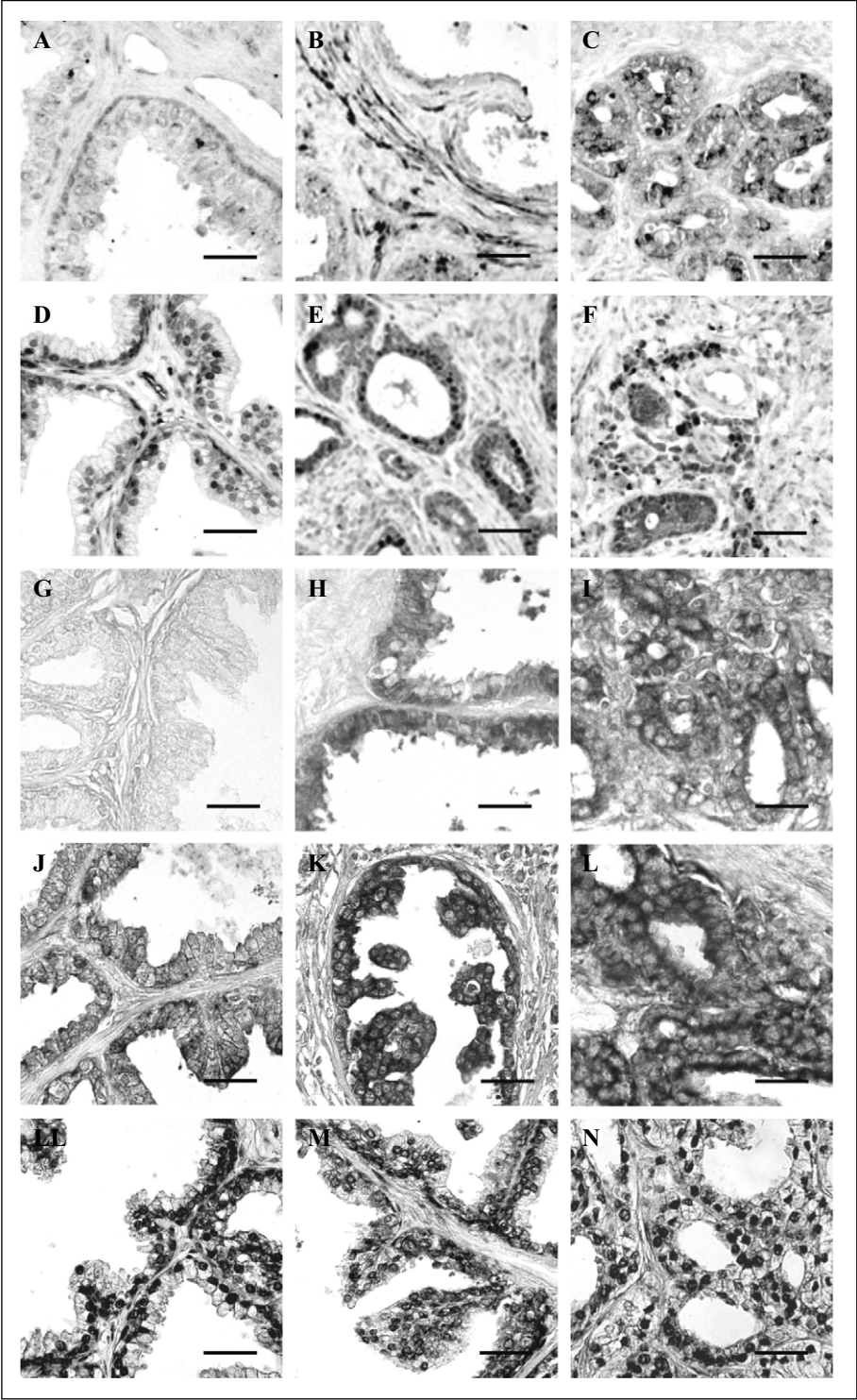


Figure 3

IL-6 was present in epithelial cells in normal prostate (A), BPH (B) and PC (C). Gp-130 immunostaining appeared in the epithelial cells of BPH (D) and PC (E-F) samples; no differences were observed between low (E), medium and high (F) Gleason scores. Raf-1 was negative in normal prostates or low-grade PIN (G), but the cytoplasm of the epithelial cells presented a positive immunoreaction in BPH (H), low-grade PIN and PC with medium Gleason score (I) samples. MEK-1 presented an immunoreaction in epithelial cells of BPH (J), high-grade PIN (K) and PC (L). P-MEK-1 presented an immunoreaction in the nuclei of epithelial cells of BPH (LL), high-grade PIN (M) and PC (N). Scale bars: 20 μm (I, L-N) and 25 μm (A-H, J-K).

p-ERK was found in the cytoplasm of epithelial cells in 40% of normal samples and low-grade PIN, 37.14% of BPH (figure 4G), 40% of high grade PIN (figure 4H), 38.09% of cancer with a low Gleason score, 37.25% of cancer with a medium Gleason score and 38.09% of

cancer with a high Gleason score (figure 4I). The lowest optical density was found in normal prostate, BPH and low- grade PIN; and the highest in PC and high grade PIN, but no differences between Gleason scores and high-grade PIN were found (table 2).

Table 1
Percentages of patients showing positive immunohistochemical reactions to IL-6, Gp-130, Raf-1, MEK-1 and p-MEK in normal prostate, benign prostatic hyperplasia (BPH), prostatic intraepithelial neoplasia (PIN) and prostatic carcinoma (PC), and average optical densities of immunostainings in positive patients

Prostate glands (no.)	IL-6		Gp-130		Raf-1		MEK-1		p-MEK	
	% of positive cases	Optical density	% of positive cases	Optical density	% of positive cases	Optical density	% of positive cases	Optical density	% of positive cases	Optical density
Normal (20)	100	6.8 ± 2.2 ^a	100	13.5 ± 2.9 ^a	0	–	0	–	40	29.77 ± 1.8 ^a
BPH (35)	80	21.8 ± 2.58 ^b	57.14	25.4 ± 2.01 ^b	37.14	19.93 ± 2.55 ^a	62.85	17.46 ± 1.64 ^a	68.57	41.22 ± 3.8 ^c
PIN										
Low-grade PIN (12)	100	5.32 ± 3.1 ^a	100	12.41 ± 2.1 ^a	0	–	0	–	41.6	28.52 ± 0.53 ^a
High-grade PIN (15)	86.6	22.6 ± 2.42 ^b	80	26.33 ± 2.51 ^b	46.6	31.18 ± 1.39 ^b	73.3	24.79 ± 1.74 ^b	73.33	35.58 ± 0.93 ^b
PC										
Low Gleason (21)	90.47	21.25 ± 2.75 ^b	80.95	27.45 ± 2.98 ^b	47.61	29.94 ± 1.87 ^b	71.42	25.79 ± 2.89 ^b	71.42	36.96 ± 2.53 ^b
Medium Gleason (51)	82.35	23.32 ± 1.78 ^b	92.15	26.2 ± 2.2 ^b	58.82	34.15 ± 2.87 ^c	56.86	31.68 ± 2.53 ^c	78.43	35.28 ± 2.65 ^b
High Gleason (21)	90.47	24.23 ± 0.91 ^b	95.23	28.92 ± 2.27 ^b	42.85	41.25 ± 2.21 ^d	47.61	38.0 ± 2.23 ^d	80.95	43.45 ± 1.5 ^c

Average optical densities were evaluated only in patients showing positive immunoreactions. Statistical analysis refers to each antibody separately. Values denoted by different superscripts are significantly different from each other. Significance was determined by the one way ANOVA test at $p \leq 0.05$, by multiple pairwise comparisons (GraphPad PRISM 3.0 computer program).

NF-κB

Very slight immunoreaction to NF-κB/p50 was localized in the cytoplasm of epithelial cells in 60% of normal prostate, 58.3% of low-grade PIN (*figure 4J*; *table 2*) and 100% of BPH patients [19]. In PC, immunostaining was more intense (higher optical density) and appeared in the cytoplasm of epithelial cells in 80.9% of low Gleason, 94.1% of medium Gleason and 100% of high Gleason scores, and in the nucleus of epithelial cells in 30.1% of patients with low, 41.2% with medium (*figure 4K*), and 48% with high Gleason scores (*figure 4L*) respectively. Similar data were observed in high-grade PIN, where immunoreaction was found in the cytoplasm (73.3% of patients) and nucleus (26.6% of patients) of epithelial cells [19].

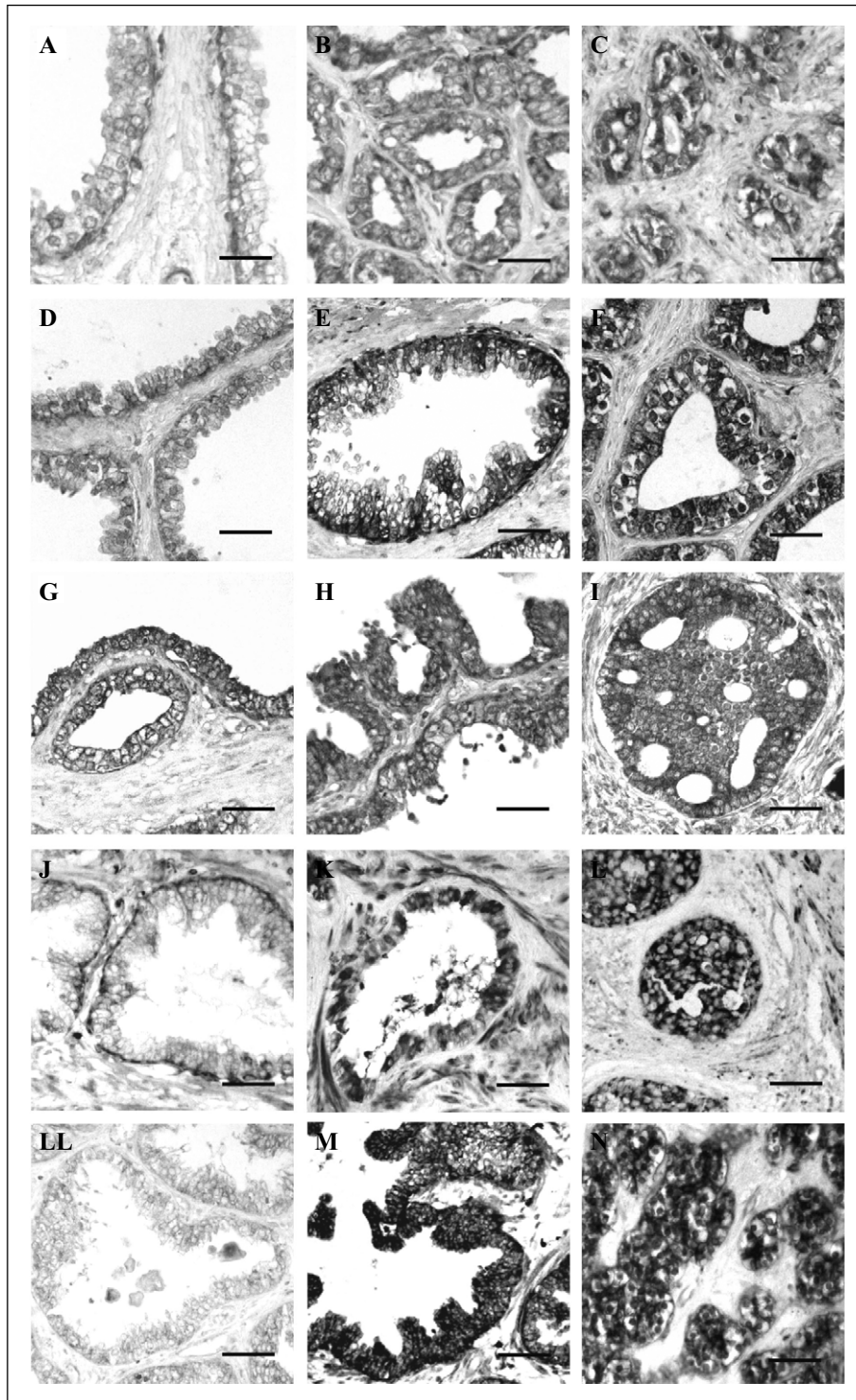
No immunoreaction to NF-κB/p65 was observed in normal prostates (*figure 4LL*) and low-grade PIN samples (*table 2*) [19]. In BPH, immunoreaction appeared in the cytoplasm of epithelial cells in 71.4% of cases. In high grade PIN (*figure 4M*), cytoplasmic (73.3%) and nuclear (13.3%) immunostainings were found in epithelial cells. In PC, cytoplasmic immunostaining was observed in 100% with low Gleason, 92.1% with medium Gleason and 80.9% with a high Gleason scores. Nuclear immunostaining was found in 19% with low, 45.1% with medium and 71.4% with high Gleason scores (*figure 4N*). Optical density was higher in PC and high-grade PIN than in BPH samples. In PC, the optical densities increased with increasing Gleason scores (*table 2*) [19].

DISCUSSION

Our previous studies in human prostate gland suggested that the TNF-α/NIK, TNF/p38, IL-1/NIK and IL-1/p38 transduction pathways induce activation of NF-κB [23, 24]. The present study provides information about the role of the IL-6/ERK transduction pathway in the activation of NF-κB, the role of NF-κB in IL-6 expression, and consequently, on the proliferation/apoptosis balance in human prostate tissue.

In the normal prostate gland, we found IL-6, gp130, ERK-1, ERK-2 and NF-κB/p50 located in the cytoplasm of epithelial cells. Immunoreactions to IL-6 and gp-130 were scant, suggesting that IL-6 is not highly active in normal tissue. No immunoreaction was found to Raf-1, MEK-1 and NF-κB/p65. The presence of p-MEK (activated) in these patients could have been triggered by TRAF-2 or TRAF-6. When MEK is activated phosphorylated ERK. p-ERK does not seem to activate NF-κB since immunoreaction to NF-κB/p50 was scanty (localized in the cytoplasm of epithelial cells) and no immunoreaction to NF-κB was found. Perhaps ERK activates other transcription factors such as Elk-1, ATF-2, Ap-1 or c-myc. Given these results, we believe that this transduction pathway may not be very active. Since in low-grade PIN, we obtained similar results to those described in normal prostate samples, this transduction pathway also could be not very active.

In BPH, all the factors studied were expressed. The optical density for p-ERK was similar to those observed

**Figure 4**

ERK-1 immunostaining appeared in the cytoplasm of epithelial cells of BPH (A) and PC (B-C) samples, immunoreaction increasing with increasing Gleason scores, of lower intensity in medium (B) than in high (C) Gleason score samples. ERK-2 showed cytoplasmic immunoreaction in epithelial cells of BPH (D), high-grade PIN (E) and PC (F). P-ERK appeared in the cytoplasm of epithelial cells of BPH (G), high grade PIN (H) and PC (I). P50 was faint in the cytoplasm epithelial cells of low-grade PIN (J), but in PC immunostaining also was nuclear, increasing the expression in medium (K) and high (L) Gleason. No immunoreaction was found to p65 in normal prostate (LL), but was localized in the cytoplasm of epithelial cells in BPH, PIN (M) and PC (N) samples; however, in PC immunoreaction was also localized in the nuclei of epithelial cells, nuclear localization increasing with increasing Gleason scores. Scale bars: 20 μ m (A-C, F), 25 μ m (D, H-L, N) and 30 μ m (E, G-J, LL-M).

Table 2
Percentages of patients showing positive immunohistochemical reactions to ERK-1, ERK-2, p-ERK, p50 and p65 in normal prostate (NP), benign prostatic hyperplasia (BPH), prostatic intraepithelial neoplasia (PIN) and prostatic carcinoma (PC), and average optical densities of immunostainings in positive patients

Prostates (no.)	ERK-1		ERK-2		p-ERK		p50		p65	
	% of positive cases	Optical density	% of positive cases	Optical density	Optical density	Optical density	Optical density	Optical density	% of positive cases	Optical density
Normal (20)	80	20.24 ± 2.6 ^a	100	30.21 ± 1.77 ^a	40	18.4 ± 0.31 ^a	60	7.88 ± 2.4 ^a	0	—
BPH (35)	80	28.99 ± 2.58 ^b	71.42	46.67 ± 2.01 ^b	37.14	19.21 ± 1.01 ^a	100	18.6 ± 1.72 ^b	71.4	9.08 ± 3.23 ^a
PIN										
- Low-grade PIN (12)	80	22.65 ± 0.88 ^a	100	30.83 ± 1.01 ^a	40	18.53 ± 0.13 ^a	58.3	15.32 ± 2.00 ^b	0	—
- High-grade PIN (15)	86.6	35.82 ± 2.33 ^c	86.6	58.77 ± 2.33 ^c	40	23 ± 0.88 ^b	73.3	27.51 ± 2.79 ^c	73.3	30.17 ± 3.08 ^c
Cytoplasm Nucleus							26.6	28.76 ± 3.31 ^ψ	13.3	29.77 ± 2.13 ^ψ
PC										
Low										
- Low Gleason (21)										
Cytoplasm	90.47	36.14 ± 2.75 ^c	80.95	58.62 ± 2.98 ^c	38.09	23.41 ± 0.92 ^b	80.9	28.01 ± 2.25 ^c	100	23.8 ± 2.93 ^b
Nucleus							30.1	32.08 ± 2.96 ^ψ	19	28.34 ± 2.04 ^ψ
- Medium Gleason (51)										
Cytoplasm	96.07	41.5 ± 1.78 ^d	82.35	59.27 ± 1.62 ^c	37.25	23.69 ± 1.81 ^b	94.1	29.17 ± 1.22 ^c	92.1	29.09 ± 1.90 ^c
Nucleus							41.2	33.82 ± 4.07 ^ψ	45.1	34.91 ± 4.32 ^ψ
- High Gleason (21)										
Cytoplasm	90.47	49.24 ± 0.91 ^c	76.19	60.92 ± 2.27 ^c	38.09	23.61 ± 2.49 ^b	100	39.37 ± 4.15 ^d	80.9	36.71 ± 2.18 ^d
Nucleus							47.6	41.26 ± 2.31 [§]	71.4	46.56 ± 1.20 [§]

Average optical densities were evaluated only in patients showing positive immunoreactions. Statistical analysis refers to each antibody separately. Values denoted by different superscripts are significantly different from each other. Significance was determined by the one way ANOVA test at $p \leq 0.05$, by multiple pairwise comparisons (GraphPad PRISMA 3.0 computer program).

in the normal sample group, but optical densities for IL-6, gp-130, Raf-1, MEK-1, ERK-1 and ERK-2 were higher compared to those described in normal prostate and low-grade PIN. At the same time, we observed immunoreaction to NF- κ B/p65, the optical density for NF- κ B/p50 being increased. Both NF- κ B subunits were localized in the cytoplasm of epithelial cells, perhaps because the activation required for translocation of NF- κ B to the nucleus was insufficient, although there was increased expression of I κ Bs [23]. In a previous paper [23], our group, with the same samples as used in the present study, described an increase in p-I κ B immunoreaction, but perhaps insufficient to activate the degradation of I κ B- α required for the translocation of NF- κ B to the nucleus. Several authors [25, 26] reported that inflammation may be a possible condition in hyperplastic prostate tissue with a risk of progression towards BPH. Under these conditions, a tissue microenvironment rich in pro-inflammatory cytokines, such as TNF- α , IL-1 or IL-6, would occur [27]. Using the same samples as used in this study, we observed not only increased levels of the cell proliferation antigen ki-67 [28], but also the pro-apoptotic TNF- α /Ap-1 transduction pathway [29], suggesting that this apoptotic pathway is activated as an attempt to inhibit uncontrolled cell proliferation (typical of BPH diseases), although this attempt is insufficient and is counteracted by other, anti-apoptotic signals such as Bcl-2 [30], p21 [31], mcl-1 [31] or transcription factors as Elk-1 and p-ATF-2 [23]. In this way, Ricote *et al.* [24] have suggested that the overexpression of these transcription factors is involved in cell proliferation and survival.

In PC, the percentage of cases showing immunoreaction to IL-6 and gp-130 decreased, but the optical densities increased to 4-5 times higher (IL-6) or 2-fold higher (gp-130). Hence, IL-6 signalling could be enhanced not only as a result of increased autocrine or paracrine production, but also because of increasing levels of this receptor. The optical densities observed for Raf-1 and MEK-1, were increased as was that for p-MEK. The expression of MEK-1 increased with the Gleason score at the same time as the percentages of positive samples decreased. Whereas the expression of p-MEK increases with the Gleason score, at the same time, the percentage of positive patients increases. These increases are insufficient to activate ERK since no variation was found in the percentage of positive patients (less than 40%) for the phosphorylated form (p-ERK), and optical density was hardly increased in PC. The NF- κ B/p50 location changes, from the cytoplasm to the nucleus in PC patients at the same time NF- κ B/p65 is also expressed in the nucleus. Shukla *et al.* [32] described in human prostate, a progressive increase in the expression of NF- κ B/p65 (but not of NF- κ B/p50) in PC compared to benign samples, and this increase correlated with the increased levels of I κ B α and its phosphorylation. When NF- κ B is located in the nucleus, it may promote cell growth and proliferation in prostate cancer cells [33] by regulating expression of genes such as IL-6 [13]. Characterization of the IL-6 promoter has revealed a complex control region with binding motifs for NF- κ B [14]. In previous studies on multiple myeloma cells [34] and astrocytes [35], the authors

propose that NF- κ B is mainly required for induced IL-6 transcription. Similar results have been described by Paule *et al.* [36] in metastatic androgen-independent prostate cancer. Immunoreaction of IL-6 in PC was increased with Gleason score (not statistically significant). The immunoreaction of NF- κ B was also increased with the Gleason score. When we compare these results, it was seen that in 64.51% of patients with high expression of NF- κ B, high expression of IL-6 is also present.

In previous studies and with the same patients as used here, we also described an increased proliferation index measured by antigen ki-67 [28]. High grade PIN is the earliest accepted stage in carcinogenesis, possessing most of the phenotypical, biochemical and genetic changes of cancer but without invasion into the fibromuscular stroma [37]. In this study, high-grade PIN and prostate cancer share similar alterations in the IL-6/ERK transduction pathway. These data suggest that in PC and high-grade PIN, nuclear translocation of NF- κ B could be poorly stimulated by the IL-6/ERK transduction pathway, but could be activated by other transduction pathway, such as TNF- α /NIK, TNF/p38, IL-1/NIK or IL-1/p38. The IL-6/ERK transduction pathway could be activating other transcription factors, related to proliferation, that are increased in PC, such as Elk-1, ATF-2 or c-myc, and that we have described in previous manuscripts [24, 38]. At the same time, overexpression of NF- κ B could be inducing endogenous IL-6 transcription, since IL-6 was also increased in cancer.

CONCLUSION

Translocation of NF- κ B to the nucleus in PC and high-grade PIN could be slightly stimulated by the IL-6/ERK transduction pathway, but could also be stimulated by other transduction pathways, triggered by other pro-inflammatory, such as TNF- α /NIK, TNF/p38, IL-1/NIK or IL-1/p38. NF- κ B has been considered to be a marker predicting PC since nuclear localization was only observed in PC. Activation of NF- κ B in PC could regulate IL-6 expression. Other transcription factors such as Elk-1, ATF-2 or c-myc, related to cell proliferation and activated by these pro-inflammatory cytokines, were also increased in PC. Therefore, since PC is a heterogeneous disease in which multiple transduction pathways may contribute to an uncontrolled apoptosis/cell proliferation balance, we conclude that significant attention should be focused on the rational combination of novel agents directed toward the inactivation of pro-inflammatory cytokines, which could disrupt complementary tumour cell proliferation pathways.

Disclosure and financial support. This study was supported by grants from the "Ministerio de Educación y Ciencia", Spain (SAF2007-61928) and the "Fundación Mutua Madrileña", Spain (AP76182010). Gonzalo Rodríguez-Berriguete and Angela Prieto were awarded a pre-doctoral fellowship from the Alcalá University (Madrid, Spain) during the course of this work.

None of the authors has any conflict of interest to disclose.

REFERENCES

1. Cerutti PA, Trump BF. Inflammation and oxidative stress in carcinogenesis. *Cancer Cells* 1991; 3: 1.
2. Hussain SP, Hofseth LJ, Harris CC. Radical causes of cancer. *Nat Rev Cancer* 2003; 3: 276.
3. Bouraoui Y, Ricote M, García-Tuñón I, *et al.* Pro-inflammatory cytokines and prostate-specific antigen in hyperplasia and human prostate cancer. *Cancer Detect Prev* 2008; 32: 23.
4. Taga T, Kishimoto T. Gp130 and the interleukin-6 family of cytokines. *Annu Rev Immunol* 2003; 15: 797.
5. Werlen G, Hausmann B, Naeher D, Palmer E. Signaling life and death in the thymus: timing is everything. *Science* 2003; 299: 1859.
6. Turjanski AG, Vaqué JP, Gutkind JS. MAP kinases and the control of nuclear events. *Oncogene* 2007; 26: 3240.
7. McCubrey JA, Steelman LS, Chappell WH, *et al.* Roles of the Raf/MEK/ERK pathway in cell growth, malignant transformation and drug resistance. *Biochim Biophys Acta* 2007; 1773: 1263.
8. Zhu Y, Culmsee C, Klumpp S, Krieglstein J. Neuroprotection by transforming growth factor-beta1 involves activation of nuclear factor-kappaB through phosphatidylinositol-3-OH kinase/Akt and mitogen-activated protein kinase-extracellular-signal regulated kinase1,2 signaling pathways. *Neuroscience* 2004; 123: 897.
9. Chu LF, Wang WT, Ghanta VK, Lin CH, Chiang YY, Hsueh CM. Ischemic brain cell-derived conditioned medium protects astrocytes against ischemia through GDNF/ERK/NF-kB signaling pathway. *Brain Res* 2008; 1239: 24.
10. Karin M. Nuclear factor-kappaB in cancer development and progression. *Nature* 2006; 441: 431.
11. Rodríguez-Berriguete G, Fraile B, de Bethencourt FR, *et al.* Role of IAPs in prostate cancer progression: immunohistochemical study in normal and pathological (benign hyperplastic, prostatic intraepithelial neoplasia and cancer) human prostate. *BMC Cancer* 2010; 10: 18.
12. Aggarwal BB. Tumour necrosis factors receptor associated signalling molecules and their role in activation of apoptosis, JNK and NF-kappaB. *Ann Rheum Dis* 2000; 59: i6-i16.
13. Suh J, Rabson AB. NF-kappaB activation in human prostate cancer: important mediator or epiphenomenon? *J Cell Biochem* 2004; 91: 100.
14. Dendorfer U, Oettgen P, Libermann TA. Multiple regulatory elements in the interleukin-6 gene mediate induction by prostaglandins, cyclic AMP, and lipopolysaccharide. *Mol Cell Biol* 1994; 14: 4443.
15. Gerlo S, Haegeman G, Vanden Berghe W. Transcriptional regulation of autocrine IL-6 expression in multiple myeloma cells. *Cell Signal* 2008; 20: 1489.
16. Sebolt-Leopold JS, Herrera R. Targeting the mitogen-activated protein kinase cascade to treat cancer. *Nat Rev Cancer* 2004; 4: 937.
17. Grant S. Cotargeting survival signaling pathways in cancer. *J Clin Invest* 2008; 118: 3003.
18. Weber MJ, Gioeli D. Ras signaling in prostate cancer progression. *J Cell Biochem* 2004; 91: 13.
19. Keller ET, Fu Z, Yeung K, Brennan M. Raf kinase inhibitor protein: a prostate cancer metastasis suppressor gene. *Cancer Lett* 2004; 207: 131.
20. Lee Jr JT, Steelman LS, McCubrey JA. Modulation of Raf/MEK/ERK kinase activity does not affect the chemoresistance profile of advanced prostate cancer cells. *Int J Oncol* 2005; 26: 1637.
21. Royuela M, Arenas MI, Bethencourt FR, Sánchez-Chapado M, Fraile B, Paniagua R. Regulation of proliferation/apoptosis equilibrium by mitogen-activated protein kinases in normal, hyperplastic, and carcinomatous human prostate. *Hum Pathol* 2002; 33: 299.
22. Royuela M, Ricote M, Parsons MS, García-Tuñón I, Paniagua R, de Miguel MP. Immunohistochemical analysis of the IL-6 family of cytokines and their receptors in benign, hyperplastic, and malignant human prostate. *J Pathol* 2004; 202: 41.
23. Nuñez C, Cansino JR, Bethencourt F, *et al.* TNF/IL-1/NIK/NF-kappa B transduction pathway: a comparative study in normal and pathological human prostate (benign hyperplasia and carcinoma). *Histopathology* 2008; 53: 166.
24. Ricote M, Garcia-Tunon I, Bethencourt F, *et al.* The p38 transduction pathway in prostatic neoplasia. *J Pathol* 2006; 208: 401.
25. Crawford ED, DeAntoni EP, Ross CA. The role of prostate-specific antigen in the chemoprevention of prostate cancer. *J Cell Biochem* 1996; 25: 149.
26. Roehrborn CG. Definition of at-risk patients: baseline variables. *BJU Int* 2006; 97: 7.
27. Mantovani A, Locati M, Vecchi A, Sozzani S, Allavena P. Decoy receptors: a strategy to regulate inflammatory cytokines and chemokines. *Trends Immunol* 2001; 22: 328.
28. de Miguel MP, Royuela M, Bethencourt FR, Santamaria L, Fraile B, Paniagua R. Immunoexpression of tumour necrosis factor- α and its receptors 1 and 2 correlates with proliferation/apoptosis equilibrium in normal, hyperplastic and prostatic carcinomatous human prostate. *Cytokine* 2000; 12: 535.
29. Ricote M, Royuela M, Garcia-Tunon I, Paniagua R, Fraile B. Pro-apoptotic tumor necrosis factor-alpha transduction pathway in normal prostate, benign prostatic hyperplasia and prostatic carcinoma. *J Urol* 2003; 170: 787.
30. Royuela M, De Miguel MP, Bethencourt FR, Fraile B, Arenas MI, Paniagua R. IL-2, its receptors, and bcl-2 and bax genes in normal, hyperplastic and carcinomatous human prostates: immunohistochemical comparative analysis. *Growth Factors* 2000; 18: 135.
31. Royuela M, Arenas MI, Bethencourt FR, Sánchez-Chapado M, Fraile B, Paniagua R. Immunoexpressions of p21, Rb, mcl-1 and bad gene products in normal, hyperplastic and carcinomatous human prostates. *Eur Cytokine Netw* 2001; 12: 654.
32. Shukla S, MacLennan GT, Fu P, *et al.* Nuclear factor-kappaB/p65 (Rel A) is constitutively activated in human prostate adenocarcinoma and correlates with disease progression. *Neoplasia* 2004; 6: 390.
33. Wu JT, Kral JG. The NF-kappaB/IkappaB signaling system: a molecular target in breast cancer therapy. *J Surg Res* 2005; 23: 158.
34. Gerlo S, Haegeman G, Vanden Berghe W. Transcriptional regulation of autocrine IL-6 expression in multiple myeloma cells. *Cell Signal* 2008; 20: 1489.
35. Spooren A, Kooijman R, Lintermans B, *et al.* Cooperation of NFkappaB and CREB to induce synergistic IL-6 expression in astrocytes. *Cell Signal* 2010; 22: 871.
36. Paule B, Terry S, Kheuang L, Soyeux P, Vacherot F, de la Taille A. The NF-kappaB/IL-6 pathway in metastatic androgen-independent prostate cancer: new therapeutic approaches? *World J Urol* 2007; 25: 477.
37. Bostwick DG, Meiers I. Neoplasms of the prostate. In: Bostwick DG, Cheng L, eds. *Urologic Surgical Pathology*. Mosby Elsevier, 2008.
38. Royuela M, Arenas MI, Bethencourt FR, Sánchez-Chapado M, Fraile B, Paniagua R. Immunoexpressions of p21, Rb, mcl-1 and bad gene products in normal, hyperplastic and carcinomatous human prostates. *Eur Cytokine Netw* 2001; 12: 654.