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ORIGINAL ARTICLE

Prostate specific antigen and NF-kB in prostatic disease: relation with malignancy

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Abstract
Introduction: NF-kB (p50/p65) is a transcription factor involved in TNF-α-induced cell death resistance by promoting several antiapoptotic genes. We intend to relate the expression of NF-kB (p50 and p65) with serum levels of prostate-specific antigen (PSA), both in normal males and in those with pathologic conditions of the prostate.
Materials and methods: This study was carried out in 5 normal, 24 benign prostatic hyperplastic (BPH) and 19 patients with prostate cancer (PC). Immunohistochemical and Western blot analyses were performed on tissue and serum PSA was assayed by PSA DPC Immulite assays (Diagnostics Products Corporation, Los Angeles, CA).
Results: In controls, p65 NF-kB was not found and p50 was scantly detected in 60% normal samples in the cytoplasm of epithelial cells. Both p50 and p65 were expressed in 62.5% of samples with BPH and in 63.2% of the samples with PC. Both increased its frequency of expression with higher PSA serum levels.
Conclusions: Activation of NF-kB revealed by its nuclear translocation in prostate cancer could be related to cancer progression and elevated serum levels of PSA. A better understanding of the biologic mechanism by which circulating PSA levels increase and its relation with NF-kB expression is needed. Possibly, NF-kB blockage could be used as a therapeutic target to counteract proliferation in prostate cancer.
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PALABRAS CLAVE
NF-kB/p50;
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Próstata;
Hiperplasia prostática benigna;
Cáncer

Antígeno prostático específico y NF-kB en patología prostática: relación con la malignidad

Resumen

Introducción: NF-kB (p50/p65) es un factor de transcripción implicado en la resistencia a muerte celular provocada por TNF-α que promueve diferentes genes antiapoptóticos. Pretendemos relacionar la expresión de NF-kB con los niveles de antígeno prostático específico (PSA) en suero, tanto en varones sanos como en los que padecen condiciones patológicas de la glándula próstatica.

Métodos: El estudio se realizó en 5 varones sanos (controles), 24 pacientes con hiperplasia benigna de próstata (HBP) y 19 pacientes con cáncer de próstata (CP). Se llevó a cabo Western blot e inmunocitoquímica en tejido y se evaluó el PSA sérico mediante *PSA DPC immulite assays* (Diagnostics Products Corporation, Los Ángeles, CA).

Resultados: En los controles no se detectó el componente p65 de NF-kB y el p50 se detectó débilmente en el 60% de las muestras en el citoplasma de células epiteliales. Tanto p50 como p65 se expresaron en el 62,5% de las muestras de HPB y en el 63,2% de los pacientes con CP. Ambos aumentaron su frecuencia de expresión a mayor nivel de PSA.

Conclusiones: La activación de NF-kB puesta en evidencia por translocación nuclear en CP parece estar estrechamente relacionada con la progresión de la enfermedad y con los niveles séricos de PSA. Se necesita un mejor conocimiento del mecanismo biológico de la elevación del PSA circulante y de su relación con la expresión de NF-kB. Tal vez el bloqueo de NF-kB podría emplearse como diana terapéutica para frenar la proliferación del cáncer de próstata.

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Introduction

Mutations and epigenetic changes in genes involved in the control of normal cell growth and tissue homeostasis result in the malignant transformation of cancer precursor cells. In addition, some factors from the tumour microenvironment which are extrinsic to the malignant cells contribute together with genetic and epigenetic changes to tumour development and progression. In many cancers, the microenvironment surrounding malignant cells is inflammatory in nature, and a link between inflammation and cancer has been stablished.[1] Prostate cancer is the malignant disease most frequently diagnosed in men and remains the second leading cause of cancer-related death. One-third of men between the ages of 30 and 40 displays histological evidence of prostatic adenocarcinoma, and in sexagenarian men from the USA, the prevalence of the disease increases to approximately 60% In view of the high incidence of prostatic malignancy and the frequent presence of signs of inflammation in the prostate, it is suspected that chronic or recurrent inflammation contributes to development of the disease.[2]

The nuclear factor-kappaB (NF-kB) plays a pivotal role in inflammation and promotes the expression of genes involved in some aspects of cancer, such as survival, proliferation, cell-cycle control, angiogenesis and invasiveness. Therefore, NF-kB could provide a mechanistic link between inflammation and cancer, as a component of signalling pathways triggered by infectious agents, pro-inflammatory cytokines (e.g. IL-1, TNF-α), growth factors and danger signals released by necrotic cells.[3]

NF-kB / Rel is a gene family that shares a Rel Homology Domain (RHD) and comprises five proteins: NF-kB1 (p50/p105), NF-kB2 (p52/p100), RelA (p65), c-Rel and RelB.[4] NF-kB1 and NF-kB2 are synthesized as precursor molecules, p105 and p100 respectively, which after proteasome-mediated processing lead to the mature forms p50 and p52.[5] Functional NF-kB transcription factors are constituted by dimers of the five members in almost any combination. Although all of them contain the DNA binding domain (RHD), only p65, c-Rel, RelB, but not p50 or p52, possess C-terminal transactivation domains. Therefore, only p50/p65, p50/c-Rel, p65/p65, and p65/c-Rel dimers all are transcriptionally active, whereas p50 homodimer and p52 homodimer are transcriptionally repressive.[6]

Canonical pathway activates dimers containing either p65 or c-Rel, together with p50. In a basal state, these dimers are retained in the cytosol by binding to the inhibitors of NF-kB (IkBs). Cell stimulation activates the IκB kinase (IKK) complex, composed of two catalytic subunits, IKK-α and IKK-β, and the regulatory subunit IKKγ. Activated IKK complex phosphorylates IκBs and targets them for polyubiquitination and degradation via proteasome. NF-κB nuclear localization signal is consequently exposed and NF-kB is translocated to the nucleus where it carries out its transcriptional activity. This pathway is initiated by pro-inflammatory cytokines, such as IL-1 and TNF-α and viral infections.[7-9]

At present, several research studies are focusing in the search for new biochemical markers capable of predicting and diagnosing prostate cancer. In addition, pro-inflammatory cytokines (such as IL-1 or IL-6) were related to the production of PSA and the progression of prostate cancer.[10-13] PSA is an organ-specific marker, with high sensitivity but low specificity for the detection of prostate cancer, there being tumour-unrelated situations that cause temporary increases.[14,15] Although dynamic variations of the marker can be useful in determining histopathologic findings of prostate cancer,[16] we can say PSA is not a good indicator of the evolution of disease.[17,18] The aim of our study was to relate the expression NF-kB (p50 and p65), analyzed by Western blot and immunohistochemistry, with serum levels of PSA in normal and pathologic prostatic tissue in order to elucidate their possible role in tumor progression. We are also discussing the possible use of NF-kB as a new therapeutic target.

Material and methods

in this study we obtained transurethral resections specimens from 25 men (aged from 55 to 85 years) with clinical and histopathologic diagnosis of benign prostate hyperplasia (BPH), radical prostatectomies from 17 men (aged from 57 to 88 years) diagnosed with prostate cancer (PC) and 5 men samples (aged from 20 to 38 years) obtained at autopsy (8-10 hours after death) without history of reproductive, endocrine or related diseases. Each sample was divided into three portions: one portion was immediately processed for immunohistochemistry and the other two portions were frozen in liquid nitrogen and maintained at –80º C for Western Blot analysis. All pathological, clinical or personal data were anonymized and separated from any personal identifiers. This study was reviewed and approved by the Military Hospital of Tunis and Hospital of Fatouma Bourguiba of Monastir (Tunisia), and was performed with the consent of the patients or their family in autopsy cases.

In order to determine serum PSA levels before the radical prostatectomy, we used the PSA DPC Immulite assays (given by the Diagnostics Products Corporation, Los Angeles, CA). These assays were performed according to the manufacturer's instructions. The primary antibodies (Santa Cruz Biotechnology, Ca, USA) were: rabbit anti-human NF-kB/p-50 and mouse anti-human NF-kB/p-65, at 1:50 (immunohistochemistry) or 1:200 (Western blot) dilutions. In Western blot, mouse anti-chicken α-actin (Amersham, Madrid, Spain) was also used at the same dilution to examine the relative expression of the other proteins. Procedure specificity was checked using negative and positive controls. For negative controls sections of different tissue groups were incubated with preimmune serum or with blocking peptides (Santa Cruz Biotechnology) at the same immunoglobulin concentration used for each antibody. As positive controls, histologic sections of skin were incubated with the same antibodies used in this study.

For each antibody a histologic quantification of immunolabeling was performed. Of each prostate, six histologic sections were selected at random. Using the X40 objective 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). For each positively immunostained section, one negative control section was also used, and the optic density of this control section was taken away from that of the stained section. After, means ± SD for each prostatic type were calculated. The same calculation was performed in normal, BPH and cancer samples. The statistical significance between means of the different prostate samples was assessed by the Fisher exact and the one-way ANOVA test at $p \leq 0.05$ (GraphPad PRISMA 3.0 computer program).

Table 1 Intensity of immunostaining (mean optical density +/-SD) in Western blot for different tissues

	NF-kB p50*	NF-kB p65*
Normal	5.76±3.7[a]	—
Hyperplasia	22.76±3.8[b]	20.32±2.4[a]
Cancer	39.67±2.7[c]	58.9±3.6[b]

*Evaluated only in patients with positive immune reaccion. Values with different superindexes significantly differ for each antibody (column) ($p \leq 0.05$).

Results

Western blot

Western blot analysis showed a single band for NF-kB/p50 at the corresponding molecular weight in NP, BPH and PC. In normal prostates (NP), NF-kB/p65 was not detected, while immunoreactions for the other antibodies were found at the corresponding molecular weight (fig. 1). Comparison of optical densities revealed significant differences among the three prostate groups (Normal, BPH, PC) (table 1).

Immunohistochemistry

No immunoreaction was observed in negative controls incubated with pre-immune serum, or using the antibodies preabsorbed with an excess of purified antigens. Skin sections used as positive controls were always positive. For each antibody assayed, the percentages of positive cases are shown in table 2.

Immunoreaction to NF-kB/p50 (fig. 2A) was found in the epithelial cell cytoplasm of NP (60%), BPH (62.5%) and PC (63.15%) patients (table 2). In BPH (fig. 2B) immunostaining was positive in 70% of samples with PSA levels between 0-4 ng/ml, 55.5% of samples with PSA level between 4-20 ng/ml and 60% of patients with PSA levels >20 ng/ml. In PC (fig. 2C), immunoreaction to NF-kB/p50 was positive in the 50%, 57.14% and 70% of patients with 0-4 ng/ml, 4-20 ng/ml and >20 ng/m respectively. Moreover, nuclear NF-kB/p50 inmmunostaining was observed in PC.

No immunoreaction was found to NF-kB/p65 in normal prostate samples (fig. 2D). In BPH patients (fig. 2E) the percentage of positive cytoplasmic staining (cytoplasmic immunoreaction) decreased as PSA levels increased (table

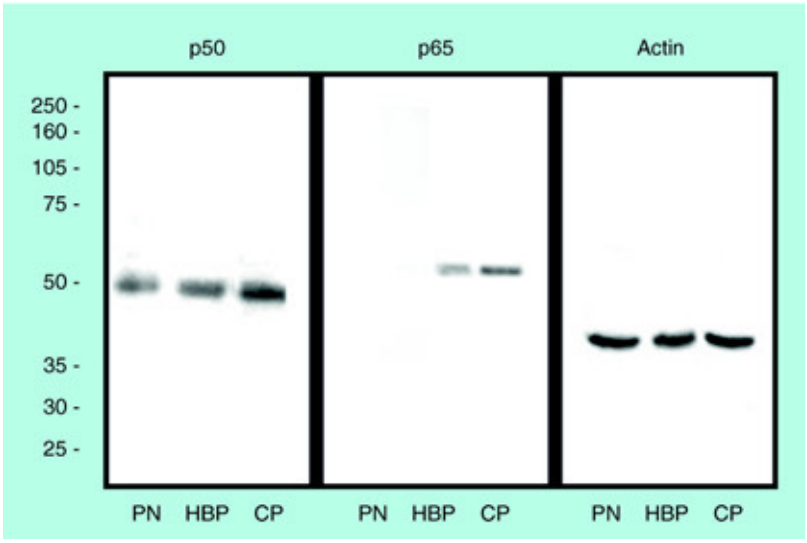

Figure 1 Western blot analysis of NF-kB/ p50 and NF-kB/ p65 after 15%polyacrylamide gel electrophoresis. NP: normal prostate. BPH: benign prostate hyperplasia. PC: prostate carcinoma. The lanes show a band corresponding to a positively stained prostate of each group, although NF-kB/ p65 was not found in NP.

2). In PC samples (fig. 2F), cytoplasmic and nuclear immunoexpressions to NF-kB/ p65 was observed in the groups with different PSA levels: 0-4 ng/ ml (50%), 4-20 ng/ ml (57.1%) and >20ng/ml (70% of patients).

Discussion

There is a growing amount of evidence supporting a link between inflammation and some malignancies. Prostate cancer and prostatitis are both frequent entities of prostatic disease.[2] However causative and mechanistic aspects connecting them are not widely understood. The transcription factor NF-kB promotes the expression of several genes implicated in inflammation, cell-cycle control, survival, proliferation, angiogenesis and invasiveness. In this way, in a chronic inflammatory microenvironment, NF-kB could act within both inflammatory and malignant cells establishing signalling loops among them which reinforce the inflammatory microenvironment and supplies surveillance signals to malignant cells.[3]

In vitro studies and experiments in some animal models have highlighted its role as tumour promoter and in resistance to anticancer drugs and radiation therapy. We focused our study on NF-kB/ p50 and NF-kB/ p65 subunits given p50/ p65 is the major complex responsible for NF-kB-regulated transcriptional activation, whereas p50/ p50 complex mainly exerts repression.[19-21]

In normal tissue samples only p50 and not p65 was detected, and its location was restricted to the cytoplasm of epithelial cells where they are not able to exert its transcriptional function. In BPH, both subunits were

Table 2 Percentage of patients with positive NF-kB (p50) and NF-kB (p65) immunohistochemistry in different tissues according to seric PSA

Niveles PSA ng/ ml	NF-kB (p50) %OD*		NF-kB (p65) %OD*	
Normal (5)				
<4 (5)	3 (60%)	8.26±2.37[a]	0 (0%)	—
Hyperplasia (24)	15 (62.5%)	18.46±2.04[b]	15 (62.5%)	18.66±1.59[a]
0-4 (10)	7 (70%)		5 (80%)	
4-20 (9)	5 (55.5%)		7 (77.7%)	
> 20 (5)	3 (60%)		3 (60%)	
Cancer (19)	12 (63.15%)	28.23±2.01[c]	12 (63.15%)	28.23±2.01[b]
		32.91±2.21[* #]		32.91±2.21[* #]
0-4 (2)	1 (50%)		1 (50%)	
4-20 (7)	4 (57.14%)		4 (57.14%)	
> 20 (10)	7 (70%)		7 (70%)	

*Evaluated only in patients with positive immune reaccion. Values with different superindexes significantly differ for each antibody (column) (p≤0,05); #Nuclear immunereaction.

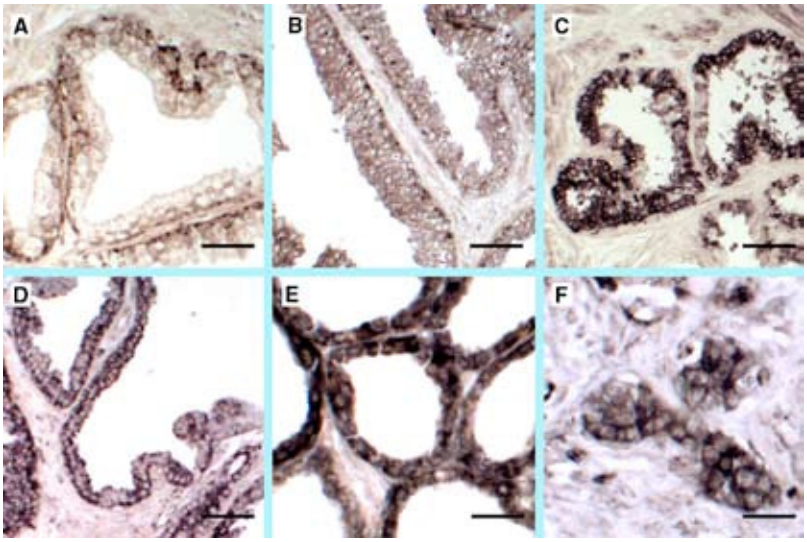

Figure 2 NF-kB/p50 was found in the cytoplasm of epithelial cells in NP (A) and BPH (B), whereas in PC also was observed in the nucleus (C). NF-kB/p65 was only observed in BPH (D) and cancer (E-F) with similar distribution to that observed to NF-kB/p50. Scale bars: 20 μm (A, E-F), 25 μm (B) and 30μm (C-D).

also localized in the epithelial cell cytoplasm as the immunoreactions increased. In PC samples, p50 as well as p65 showed an increase in both percentage and intensity of positive nuclear and cytoplasmic immunostaining. Nuclear and cytoplasmic location of NF-kB/p65 has been described by other authors, although their results were discrepant when its utility as a prognostic marker was evaluated.[22-25] Differences on experimental procedures, patient groups, antibodies used and/or patient populations could explain the discrepancies between all of these results. Our results indicate which nuclear and cytoplasmic levels of p50 and p65 are linked to progression of the prostatic malignance.[26,27] However their functional relevance in prostate cancer development remains unclear. Whereas p65/p50 and p65/p65 dimers promote the expression of NF-kB target genes, p50/p50 dimers act as transcriptional repressors. The increase in p50 nuclear expression with malignance could be explained by the translocation of p50/p50 as well as p65/p50 dimers. Therefore, p50 nuclear translocation could be involved in the repression or promotion of transcriptional activity. On the other hand, p65 nuclear translocation presumably only implies transcriptional activation. NF-kB binding activity has been reported to increase with tumor grade, and the mainly complex responsible for such as activity consisted of p50/p65 dimers.[28] In line with ours, these findings suggest that the nuclear immunostaining of p50 and p65 is mainly due to the translocation of p50/p65 dimers, which predominantly boost the transcription of NF-kB-responsive genes.

Chen et al. described in LNCaP and DU-145 human prostate cancer cell lines that NF-kB favors prostate cancer progression because it is required to activate PSA transcription.[29] This relationship between serum PSA levels and NF-kB expression could not be confirmed by Shukla et al. in studies performed *in vivo*.[28] Our results are in agreement with that obtained by Chen et al.[29] Translocation of NF-kB to the nucleus in PC might be due the overexpression of several components of the IL-1/NIK/NF-kB or TNF/NF-kB (NIK or p38) pathways. IL-1α or TNFα are the most common cytokines related to inflammation, and these cytokines are increased in cancer directly activating intermediaries of p38 pathways.[7,26,30] Recently, Bouraoui et al. described an association between the high expression of pro-inflammatory cytokines (IL-1α or IL-6), elevated PSA serum levels and tumor progression.[13] Nuclear localization of NF-kB was observed only in PC patients, it may be considered as a marker of the disease.

In conclusion, in CP was an association between the high expression of pro-inflammatory cytokines (such as IL-6 or IL-1), elevated PSA serum levels and tumor progression. These cytokines promote signaling pathways that activate several transcription factors related to survival and proliferation as NF-kB/p50 and NF-kB/p65. A better understanding of the biologic mechanism of the increase in circulating PSA levels and its relation with NF-kB expression could help to understand the evolution of this disease. Thus, NF-kB blockage could counteract the excessive proliferation index typical of prostate cancer.

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