The Role of the Non-Canonical NF-κB Pathway in Benign Prostatic Hyperplasia

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BRIEF. The role of the non-canonical NF-κB pathway was investigated by determining its effects on cell proliferation and target gene expression.

ABSTRACT. NF-κB is an important molecule that signals through canonical (p65) and non-canonical (p52) pathways. The canonical pathway can elicit pro-inflammatory factors and exacerbate many diseases, including prostate cancer. However, little is known regarding the role of the non-canonical pathway in disease. Expression of p52, a molecule associated with the non-canonical pathway, was assessed in normal prostate and benign prostatic hyperplasia (BPH) tissue samples. A DNA construct containing p52 and green fluorescent protein (GFP) was created. Human benign prostatic fibroblasts (BHPrS) and epithelial cells from BPH tissue (BPH1) were transduced with either an empty vector construct or the p52 construct (BHPrS^{-p52} and BPH1^{-p52}). Analysis of results showed increased expression of p52 in BPH tissue compared to normal prostate. BHPrS^{-p52} and BPH1^{-p52} cells proliferated faster than control cells. Expression of differentiation markers vimentin and αSMA was impaired in BHPrS; expression of possible targets of the non-canonical pathway increased. Activation of the non-canonical NF-κB pathway in prostate cells resulted in changes consistent with an ability to promote the progression of proliferative conditions such as BPH. These data have implications that should be considered as part of a more complete description of BPH development.

INTRODUCTION.

Aging correlates with the development of several benign diseases. Benign diseases, in contrast to cancer, do not invade other organs or metastasize. However, if left untreated, they can impair the quality of life of those they affect and become an economic burden. In men, benign prostatic hyperplasia (BPH) is the most prevalent disease that affects the prostate [*1*]. Similar to prostate cancer, BPH involves an excessive proliferation of cells, but it does not show signs of malignant transformation, which is characterized by invasion. Although BPH rarely causes problems before age 40, a majority of men older than 60 show histopathological evidence of disease [*2*]. Many of these men develop symptoms that require treatment [*3*]. The most common symptoms of BPH are attributed to obstruction of the urethra and loss of bladder function, leading to problems with urination [*4*].

The cause of BPH has not been completely determined, but there are several well-founded concepts relating to its origin. Because BPH mainly occurs in older men [over 50], it has been suspected to be related to aging [*5*]. Some animal studies have shown that this shift in hormone balance causes increased proliferation of prostate cells with resultant prostate enlargement [*6*]. Other possible reasons for development of BPH are insulin resistance with secondary hyperinsulinaemia (associated with type 2 diabetes), increased sympathetic nerve activity, and inflammation [*7*]. It has been suggested that inflammation leads to tissue damage and a state of chronic wound healing, which may then lead to prostate enlargement [*8*].

Activation of the nuclear factor-kappa B (NF-κB) pathway is a crucial event in many diseases, including BPH, that have an important inflammatory component [*9*]. NF-κB target genes encode proteins and microRNAs that can regulate the production of pro-inflammatory cytokines and chemokines [*10*]. NF-κB also plays a part in regulating cell survival, proliferation, and cell adhesion; therefore, it could have a significant role in the development of BPH [*10*]. There are two main pathways of NF-κB: canonical and non-canonical (Figure S1). In the canonical pathway, the IKK complex phosphorylates an IκB inhibitor, leading to the inhibitor's proteasomal degradation. The RelA/p50 dimer that the IκB inhibitor was originally attached to then translocates to the nucleus to activate target genes [*11*]. In the non-canonical pathway, NIK phosphorylates an IKKα complex that in turn phosphorylates the RelB/p100 dimer. Subsequent processing of p100 releases the RelB/p52 dimer and allows it to translocate to the nucleus to activate target genes such as CCL13, CCL19, and CCL21 [*12, 13*].

Genetic evidence suggests that the non-canonical pathway regulates important biological functions, such as lymphoid organogenesis, B-cell survival and maturation, dendritic cell activation, and bone metabolism. Deregulated non-canonical NF-κB signaling is associated with lymphoid malignancies [*11*]. Although recent works have focused on the role of p52 in prostate cancer cells, this project is the first investigation into the effects of p52 and the non-canonical pathway on benign prostate cells [*14*]. Understanding the pro-inflammatory actions of both NF-κB pathways could be beneficial for the development of new therapeutic strategies against prostate diseases.

MATERIALS AND METHODS.

Immunohistochemistry (IHC).

Prostate tissues from a tissue bank were previously fixed in 4% formalin overnight and processed to obtain paraffin blocks. Conventional IHC staining was performed as previously described [*15*]. Staining slides were de-paraffinized with histoclear and rehydrated through a series of graded ethanol solutions (from 100% to 50%). Primary antibodies p65 (Cell Signaling) at 1:200 and p100/p52 (Cell Signaling) at 1:200 were used.

Generation of the Retroviral Construct and Transduction.

The 400bp p52 gene was extracted from the p52 cFlag pcDNA3 vector (Addgene plasmid 20019) by digestion using restriction enzymes BglII and EcoRI. The DNA fragment was then inserted into the multicloning site of the pBMN-IRES-GFP retroviral vector (Figure S2). Next, the pBMN-IRES-GFP^{-p52} vector and its empty vector control pBMN-IRES-GFP^{-EV} were separately transduced into BHPrS and BPH1 cell lines and FACS sorted to enrich for GFP-positive cells. Transduction was performed by exposing cells to viral particles generated by PhoenixA cells after transfection with the plasmids.

Cell Culture and Proliferation Assay.

BHPrS and BPH1 cells were cultured in 5% FBS-RPMI media (Atlanta Biologicals) and 1% antibiotic-antimycotic (Life Technologies). BPH1 is a cell line that was previously isolated from a patient with BPH. These epithelial cells have been shown to have the ability to form benign glandular structures in combination with mesenchymal cells. BPH1 cells can also become tumorigenic under the influence of fibroblasts isolated from cancer patients [*16*]. BHPrS was previously isolated from a benign prostate surgical sample [*15*].

Approximately 1000 cells/well in a 96 well plate were seeded in triplicate in complete serum. On Days 0, 2, and 4, medium was aspirated, and cells were fixed in methanol and stained with DAPI (Vector Laboratories). Pictures were taken in three different fields and cells were counted using ImageJ. Counts for each field were averaged for each sample. Fold changes compared to Day 0 were quantified.

Western Blot.

Approximately 500,000 cells were plated in 10 cm dishes and allowed to grow until they reached 80% confluence. Isolation of protein lysates and western blot analysis was performed as previously described [*15*]. The following primary antibodies were used: p65 (Cell Signaling) at 1:1000, p100/p52 (Cell Signaling) at 1:1000, GFP (SantaCruz) at 1:5000, and ß-actin (Sigma) at 1:10000. Amersham ECL plus detection reagent was used to visualize protein bands.

Immunofluorescence.

Approximately 10,000 cells/well were plated in an 8-chamber slide and cultured for 48 hr. Then, cells were fixed with methanol, washed with PBS, and blocked for 30 min. The slides were incubated overnight at 4 °C in the following primary antibodies: p65, p100, and RelB, all at a concentration of 1:100. The next day, the primary antibodies were washed and replaced with AF488 goat anti-rabbit at 1:1000 for 1 hr. The slides were washed, mounted with media containing DAPI, and visualized under a fluorescent microscope.

RNA Isolation and Quantitative Reverse Transcriptase PCR (RT-PCR).

RNA from each cell type (BPH1 and BHPrS) was isolated in triplicate using the RNeasy kit (Qiagen) and reverse transcribed using iScript (Bio-Rad). Quantitative RT-PCR was then performed using iQTM SYBR® Green Supermix (Bio-Rad). Relative expression to the control group was calculated after data normalization using housekeeping gene GAPDH. Data was analyzed in CFX Manager™ (Bio-Rad). Candidate genes were screened (Table S1).

RESULTS.

Expression of NF-κB Pathways in Human Prostate Tissues.

Both canonical (p65) and non-canonical NF-κB (p52) expression were elevated in BPH, compared to normal prostate tissues (Figure 1A). Increased expression of p100/p52 was seen mostly in stromal cells in BPH tissues, though it also appeared in some basal cells in the epithelial compartment. Increased expression of p65 was seen in both stromal and epithelial cells in BPH.

Generation of p52-Overexpressing Prostate Epithelial and Stromal Cell Lines.

Western blots were performed on protein extracts from cell lines BPH1^{-p52} and BHPrS^{-p52} to confirm their overexpression of p52 (Figure 1B). Although expression of the NF-κB2 precursor p100 was similar for all cell lines, BHPrS^{-p52} showed increased expression of mature p52 as compared to control BHPrS. BPH1^{-p52} showed no considerable increase in p52 expression as compared to control BPH1. GFP expression confirmed the presence of the transfected cDNA construct in the p52-overexpressing cell lines. Expression of p65 remained unchanged in all cells.

Immunofluorescence staining was performed to further confirm cellular localization of p52 (Figure 1C). The non-canonical NF-κB pathway is activated when p52 and RelB are found in the nucleus. The BPH1^{-p52} and BHPrS^{-p52} cell lines showed greater nuclear localization of both p52 and RelB than their control counterparts BPH1 and BHPrS.

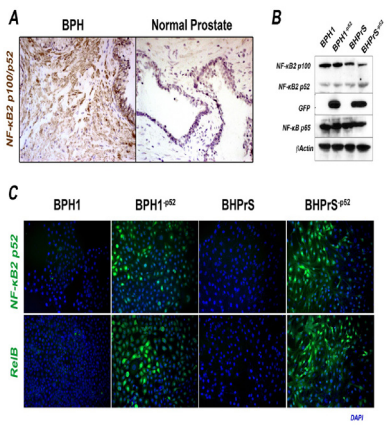

Figure 1. A. Immunohistochemical analysis of p52 expression in human prostate showed increased expression of the non-canonical NF-κB pathway in BPH-affected tissue. B. Western blot analysis showed that BHPrS^{-p52} expressed higher amounts of p52 than control BHPrS, but differences in p52 expression between BPH1^{-p52} and control BPH1 were unclear. These results were seen in two of three western blots run. Amounts of p65, p100, and β-actin remained constant between control and p52-overexpressing cell lines. C. Immunofluorescence staining showed increased nuclear localization of p52 and RelB in the p52-overexpressing cell lines.

Proliferation Assay.

BPH1^{-p52} cells proliferated at a faster rate (29.7 fold compared to D0) than control BPH1 cells did (16.4 fold compared to D0) (Figure 2A). Similarly, BHPrS^{-p52} cells proliferated at a faster rate (12.8 fold compared to D0) than control BHPrS cells did (8.1 fold compared to D0) (Figure 2B). All four cell lines showed similar general trends of growth. Soon after the fourth day, the cells reached confluence.

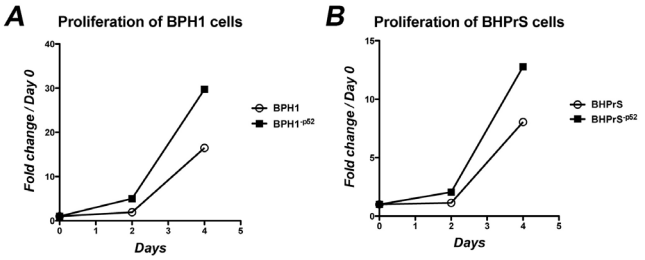

Figure 2. A. and B. Proliferation assays indicated that p52 overexpression results in a faster rate of cell growth for both BPH1 and BHPrS.

Quantitative RT-PCR.

For BPH1 cells, overexpression of p52 resulted in upregulation of CCL13, CCL19, CCL21, ICAM, and COX2 (Figure 3A). There was slight upregulation of AR and substantial upregulation of ARV7. There was little to no expression of stromal markers αSMA and vimentin in epithelial BPH1 cells. For BHPrS cells, overexpression of p52 led to downregulation of AR, ARV7, αSMA, vimentin, and COX2 (Figure 3B). Conversely, it led to upregulation of CCL13, CCL19, CCL21, ICAM, BAFF, SDF1α, TGFβ1, CCR2, and CCR7. For BPH1 cells, ICAM showed the greatest fold change (>1500 fold) when compared between control cells and p52-overexpressing cells. For BHPrS cells, CCR2 showed the greatest fold change (22 fold) when compared between control cells and p52-overexpressing cells.

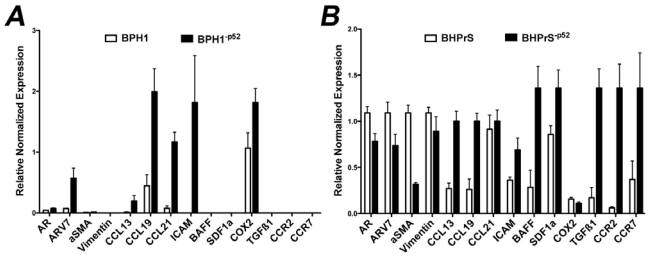

Figure 3. A. and B. Expression of putative non-canonical NF-κB target genes in BPH1^{-p52} and BHPrS^{-p52} cells, and their control counterparts.

DISCUSSION.

Previous studies have shown that stimulation of the non-canonical NF-κB pathway has a positive effect on the growth of prostate cancer cells [*14*]. Results from this project suggest that activation of p52, and therefore the non-canonical NF-κB pathway, causes an increased rate of proliferation not only in prostate epithelial cells but also in stromal cells. In this study, benign human stromal cells (BHPrS) and non-tumorigenic but initiated human epithelial cells (BPH1) were used. BHPrS cells can be "activated" by overexpressing chemokines; the activation of fibroblasts is a common feature of benign and malignant diseases such as BPH and prostate cancer [*15*].

To evaluate the status of the canonical and non-canonical NF-κB pathways in human prostate samples, paraffin-embedded tissues from patients who underwent surgical removal of their prostates were analyzed by IHC. Normal prostate tissues were excised from non-diseased areas of patients who underwent surgery due to prostate cancer. Both canonical p65 expression and non-canonical p52 expres-

sion were increased in the specimens with BPH, as compared to normal specimens. However, expression of p52 was localized more in the stromal compartment and less in the epithelial compartment. These results suggest that activation of p52 may be most common in stromal cells, which represent the major tissue involved in BPH.

Overexpression of p52 increased the proliferation of both epithelial BPH1 cells and stromal BHPrS cells. For the BPH1 cells, this change may be a reflection of the tumorigenic potential of the cell line, as upregulation of p52 in prostate cancer cell lines is also correlated with increased cell proliferation and colony formation [*14*].

Using quantitative RT-PCR, differences between control cells and p52-overexpressing cells in their expression of putative p52 target genes were measured. Expression of αSMA, a marker of smooth muscle and myofibroblast differentiation, and vimentin, a marker of fibroblasts, was decreased in p52-overexpressing BHPrS cells. Thus, activation of the non-canonical NF-κB pathway may be important for the proliferation of stromal cells before they mature along a myofibroblastic lineage.

In BHPrS cells, there was increased expression of CCL13, CCL19, and CCL21, and their corresponding receptors CCR2 and CCR7, all of which are involved in the chemotaxis of lymphoid cells to tissues. Overexpression of p52 in BHPrS cells also led to increased expression of ICAM, which is involved in lymphocyte adhesion and T-cell co-stimulation. In combination, this suggests that p52 promotes the ability of cells in not only proliferation but also the recruitment of immune cells. BPH1 cells showed increased expression of COX2, which is involved in inflammation and has been shown to be important in BPH. This has been corroborated by other studies showing that NF-κB induces expression of COX2 and cell adhesion molecules such as ICAM [*17*]. BPH is associated with inflammation, and overexpression of p52 led to increases in the expression of genes associated with inflammatory response. NF-κB is broadly classified as a response to a variety of stimuli and may be responsible for causing BPH, perhaps through mechanisms triggered by common concomitant diseases such as diabetes and obesity, and local promotion of prostatic growth. These data therefore are consistent with the hypothesis that there may be a link between p52 and BPH development and progression. Future studies on the biological (in vitro or in vivo) effects will further clarify this relationship.

Additionally, expression of TGFβ1 was increased in BHPrS^{-p52} cells. TGFβ1 has been implicated in the development and progression of BPH and prostate cancer. Expression of both BAFF, which is involved in B-cell development and survival, and SDF1α, which is involved in myeloiesis and B-cell lymphopoiesis, was increased in BHPrS^{-p52} cells. The involvement of these two factors in BPH has not been explored previously, and ongoing studies will clarify their roles.

In BPH1^{-p52} cells, there was increased expression of the constitutively active ARV7. However, expression of AR increased only slightly. The role of ARV7 is currently unknown in BPH, but it has been shown to be important in castration-resistant prostate cancer progression [*18*]. Therefore, the biological significance of this finding has yet to be explored. Conversely, BHPrS^{-p52} cells showed decreased expression of AR and ARV7 compared to control cells. This suggests that the non-canonical NF-κB pathway may influence the differentiation of epithelial and stromal cells, perhaps through the regulation of AR.

In conclusion, this project has shown for the first time that increased activation of the non-canonical NF-κB pathway by p52 in benign prostate stromal and epithelial cells produces changes consistent with those that can be found in clinical BPH. As the number of patients who suffer from the failure to control BPH increases, the need to find novel mechanisms to target in preventing BPH progression becomes greater. The information obtained in this study may be important for the development of new therapeutic alternatives to treat this widespread disease.

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SUPPORTING INFORMATION.

Supplemental Methods.

Figure S1. Canonical pathway: The IKK complex phosphorylates an IκB inhibitor, leading to the latter's proteasomal degradation. The RelA/p50 dimer then translocates to the nucleus to activate target genes. Non-canonical pathway: NIK phosphorylates an IKKα complex that then phosphorylates the IκB domain of p100. Processing of p100 results in the RelB/p52 dimer, which translocates to the nucleus to activate target genes.

Figure S2. The 400bp p52-flag DNA fragment was extracted from the parental vector by digestion using restriction enzymes BglII and EcoRI, and then inserted into the multicloning site of the pBMN-IRES-GFP retroviral vector.

Table S1. Description of the p52 candidate genes screened.

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