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RelB Enhances Prostate Cancer Growth: Implications for the Role of the Nuclear Factor- κ B Alternative Pathway in Tumorigenicity

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Abstract

The nuclear factor- κ B (NF- κ B) classic pathway is thought to be critical for tumorigenesis, but little is known about the role of the NF- κ B alternative pathway in cancer development. Recently, high constitutive nuclear levels of RelB have been observed in human prostate cancer specimens with high Gleason scores. Here, we used four complementary approaches to test whether RelB contributes to tumorigenicity of prostate cancer. Inhibiting RelB in aggressive androgen-independent PC-3 cells by stable or conditional expression of a dominant-negative p100 mutant significantly reduced the incidence and growth rate of tumors. The decrease in tumorigenicity coincided with a reduction in the NF- κ B target interleukin-8 (IL-8). Consistently, down-regulation of RelB by small interfering RNA targeting also reduced tumor growth and decreased levels of IL-8. Conversely, stable expression of RelB in androgen-responsive LNCaP tumors increased the circulating IL-8 levels. Taken together, these results reveal a tumor-supportive role of RelB, implicate the NF- κ B alternative pathway as a potential target for preventing prostate cancer, and suggest the use of IL-8 as a marker for prostate cancer prognosis.

Introduction

Nuclear factor- κ B (NF- κ B), a transcription factor, has been shown to activate numerous genes involved in tumorigenesis (1). Activation of NF- κ B in mammalian cells depends on two major NF- κ B pathways: the p50:RelA dimer-mediated classic pathway and the p52:RelB dimer-mediated alternative pathway (1,2). NF- κ B is normally sequestered in cytoplasm by its association with inhibitor proteins, such as the I κ B family members that function sequentially to conduct sustained inhibition of the classic pathway via the ubiquitination and proteasome-mediated degradation pathway (3). In addition, NF- κ B is also subject to activation by mechanisms that involve the processing of p105 and p100 to release p50 and p52, respectively (1,2).

Although the role of the classic pathway in cancer is well documented (4,5), the relationship between the alternative pathway and cancer remains to be elucidated. Prostate cancer is a

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common disease in North America (6), and as it has become more aggressive, it has acquired several new capabilities, such as self-sufficient growth signaling, insensitivity to antigrowth signals, and tumor invasion and metastasis (7). Recent studies have shown that nuclear RelB is present at a high level in prostate cancers with high Gleason scores (8). However, whether RelB contributes to progression of prostate cancer is unknown. The present study addresses this critical issue using isogenic human prostate cancer cell lines and conditional suppression of RelB in xenograft and orthotopic models. The results reveal that RelB plays an important role for prostate cancer growth *in vivo*, suggesting that the NF- κ B alternative pathway contributes to the progression of prostate cancer.

Materials and Methods

Stable cell transfection

LNCaP and PC-3 cells were grown in RPMI 1640 containing 10% FCS (Invitrogen). Plasmids to express p100 mutant (p100M; ref. 9), RelB (American Type Culture Collection), and RelB small interfering RNA (siRNA; ref. 10) were digested with *ScaI* and stably transfected into LNCaP or PC-3 cells using Lipofectamine. To control the expression level of p100M, the p100M was subcloned into a Tet-off vector (Clontech) and then stably transfected into PC-3 cells according to the manufacturer's protocol. Each stable clone was selected for Geneticin resistance and verified by Western blots.

Animals

Four-week-old male NCRNU (*nu/nu* athymic nude) mice were purchased from Taconic. In a mouse xenograft tumor model, 1.8×10^6 cells mixed with Matrigel (BD Biosciences) were injected into the right flanks of the mice. Mice injected with the p100M Tet-off clone were separated into two groups. Two days after injection, one group of mice started to drink water containing 2 mg/L doxycycline and the control group continued to drink regular water. Tumor volumes were calculated weekly using a standard formula ($A \times B^2 \times 0.52$; A and B represent the diagonal tumor lengths). In the orthotopic tumor model, the selected clones were implanted into prostate glands. At the indicated time after tumor implantation, blood samples were collected using Microtainer tubes containing lithium heparin (Becton Dickinson) and centrifuged according to the manufacturer's recommendation. Prostate-specific antigen (PSA) was quantified using a PSA ELISA kit (DRG) and interleukin-8 (IL-8) was measured by the Cytokine Core Laboratory (University of Maryland). Primary and metastatic tumors were fixed in formalin and embedded in paraffin, and 5- μ m sections were stained with H&E. Tumor incidence, size, and number of mice with metastatic lesions were analyzed in a blinded fashion by a pathologist (T.D.O.).

Western blots

Proteins extracted from cytoplasm and nuclei and Western blot procedures have been described previously (11).

NF- κ B binding assay

Nuclear proteins were used for quantifying DNA binding activities of the NF- κ B family members using an ELISA-based Trans NF- κ B Family kit (Active Motif).

Real-time PCR

To isolate total RNA, 100 mg tumor tissues were homogenized in 1 mL of Trizol reagent (Invitrogen), extracted with chloroform, and treated with a Turbo DNA-free kit (Ambion) to remove DNA. Reverse transcription reaction was performed using SuperScript First-Strand

Synthesis System for real-time PCR (RT-PCR; Invitrogen). RT-PCR was performed with gene-specific primer-probe sets using a LightCycler 480 RT-PCR System (Roche).

Soft agar assay

Culture dishes were prepared using standard medium containing 6% agar. A top agar was prepared using medium containing 0.3% agar. Cells were mixed with the top agar at a density of 10^4 /mL. Cell mixtures were added to the culture dishes and grown for 3 to 4 wk, after which colonies were counted.

Statistical data analyses

Multiple independent experiments were performed for each set of data presented. PCR and Western blots were quantified using the quantitative image analysis software Quantity One (Bio-Rad). Statistical significance was analyzed using one-way ANOVA and Tukey's multiple comparison test followed by data analysis with GraphPad Prism.

Results and Discussion

Preventing RelB nuclear translocation reduces tumorigenicity of prostate cancer cells

As the alternative pathway is activated, NF- κ B-inducible kinase (NIK) or I κ B kinase α (IKK α) phosphorylates p100, leading to ubiquitin- and proteasome-mediated p100 degradation and the release of p52 (12). A p100M expression construct that is unresponsive for NIK-dependent phosphorylation (9) was stably transfected into PC-3 cells to test whether p100M suppresses cell tumorigenicity. Selected clones were characterized by showing lower nuclear RelB levels than the control clones (Fig. 1A and B). Clones were s.c. injected into the flanks of nude male mice to verify tumorigenicity. Tumor growth rate change was quantified by counting the days required for tumor size to reach a volume of 300 mm³. Figure 1C shows that the average time for the control group to reach 300 mm³ was 24 ± 6.7 days. Tumor growth was delayed in all p100M-expressing clones: 56 ± 5.6 days for PP3 and 39 ± 18.7 days for PP10. Some tumors derived from the p100M-expressing clones (PP3) did not grow. Tumor incidence and sizes were significantly lower in the p100M-expressing clones than in the control clones (Fig. 1D).

To further test the role of RelB in tumorigenicity, p100M was expressed in a Tet-off system to allow control of RelB level in the nucleus following tumor growth in nude mice. A stable, double-transfected clone expressing p100M was controlled by administering doxycycline, which led to lower levels of RelB in the nucleus (Fig. 2A and B). The selected p100M Tet-off clone was injected into the flanks of nude mice to allow tumor growth. To suppress the effect of p100M, doxycycline was added to the drinking water. Tumor growth in the doxycycline-drinking mice was faster than in the control mice (Fig. 2C). p100M and IL-8 in tumor tissues were quantified by RT-PCR and Western blots. Levels of p100M were lower, but IL-8 levels were higher in the doxycycline-treated group than in the untreated group (Fig. 2D).

Blockage of p50:RelA dimer nuclear translocation by ablating expression or inhibition of IKK β using SN50 has been shown to significantly suppress tumorigenesis and improve therapeutic efficacy in several cancer types (13–15). Recently, we showed that prevention of p52:RelB nuclear translocation by expressing p100M and treating cells with SN52 and 1 α ,25-dihydroxyvitamin D₃ dramatically enhances radiosensitization effect (11,16,17). The present study shows that prevention of RelB nuclear translocation decreases the tumorigenicity of prostate cancer cells. Together, these results suggest that inhibition of RelB nuclear translocation can suppress tumorigenesis and radioresistance of prostate cancer.

Knockdown of RelB expression using siRNA suppresses tumorigenicity of PC-3 cells

Gene knockdown based on siRNA was used to confirm the role of RelB in tumorigenesis of prostate cancer. A plasmid-based RelB siRNA and a control siRNA were stably transfected in PC-3 cells to specifically knock down the constitutive level of RelB. Levels of RelB were lower in the selected clones (Fig. 3A). The clone was orthotopically implanted into the prostates of nude mice. Plasma samples were collected from the mice to measure production of IL-8 as a marker of tumor growth *in vivo*. The results show that IL-8 levels increased with time in the control siRNA group, but the increase was significantly lower in the RelB siRNA group (Fig. 3B). At 45 days after implantation, the prostates with tumors were excised and examined in a blinded fashion by a pathologist (T.D.O.). The results indicate that incidences of both primary carcinoma and metastasis in the control siRNA group were significantly higher than in the RelB siRNA group. In addition, sizes of primary carcinomas in the RelB siRNA group were smaller than in the control (Fig. 3C). These results confirm a tumor-supportive role of RelB and show the contribution of the alternative pathway in tumorigenesis.

Overexpression of RelB increases tumorigenicity of LNCaP cells

Because RelB level is lower in LNCaP cells than in PC-3 cells (11,16), a RelB expression construct was stably transfected into LNCaP cells to further confirm the relationship between RelB and tumorigenesis. RelB levels were higher in the RelB-overexpressing cells (Fig. 4A), which resulted in more colonies formed in soft agar than in the control (Fig. 4B). The selected clones were orthotopically implanted into the prostates of nude mice and plasmas were collected to quantify PSA and IL-8. Interestingly, PSA levels were lower in the RelB-overexpressing group, whereas IL-8 levels were higher in the RelB-overexpressing group than in the control group (Fig. 4C). In the excised prostate tumors, levels of RelB but not RelA were higher in the RelB-expressing tumors than in the control tumors. As RelB expressing, levels of IL-8 increased as expected. Unexpectedly, levels of PSA decreased in the RelB-expressing tumors compared with the control tumors (Fig. 4D).

IL-8 is constitutively expressed in many types of metastatic tumors (18). IL-8 is positively regulated by the NF- κ B pathway (19), but it is negatively regulated by androgen signaling (20). In fact, although undetectable in LNCaP cells, IL-8 is present at high levels in PC-3 cells. Our finding that tumors produced less PSA and more IL-8 as RelB level increased suggests that IL-8 is a potential molecular marker for the progression to androgen insensitivity of prostate cancer. Thus, clinical testing for recurrent tumor growth should involve measuring levels of IL-8 in addition to using the standard PSA test. Combining these tests may enhance accurate cancer detection and prognosis.

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Figure 1.

Suppression of PC-3 cell tumorigenicity as a result of p100M expression. A plasmid encoding p100M and empty vector were stably transfected in PC-3 cells and three clones (vector, PP3, and PP10) were selected. **A**, expression of p100M in PC-3 cells was confirmed by Western blots with a p52 antibody. **B**, tumors generated by injecting mice with the clones were excised and levels of RelB in the nucleus were determined by Western blots. β-tubulin and proliferating cell nuclear antigen (PCNA) were used as loading controls. **C** and **D**, after injection, tumor volumes were measured every week starting from 20 d until 60 d. Tumor growth rates and incidences were indicated. Numbers of mice: vector (15), PP3 (10), and PP10 (10). Average tumor sizes of the three groups at 52 d after tumor injections were shown above corresponding bars in **D**.

Figure 2.

Altering tumorigenicity of PC-3 cells by regulating p100M expression. p100M was subcloned into a Tet-off vector and stably transfected into PC-3 cells. *A*, nuclear and cytoplasmic proteins were extracted from the selected clones and protein levels of the NF-κB family members were measured by Western blots. β-Actin and PCNA were used as loading controls. *B*, the nuclear extracts were analyzed using a NF-κB DNA binding detection kit to determine DNA binding activities of RelA and RelB. *C*, after injection of Tet-off clones into mice, tumor size was measured on the indicated days. *D*, total RNA and proteins were extracted from the excised tumors and quantified by RT-PCR and Western blots, respectively. *Insets*, amounts of PCR products and corresponding protein levels. *Dox*, doxycycline.

Figure 3. Suppression of PC-3 cell tumorigenicity by knockdown of RelB. A plasmid-based RelB siRNA and control siRNA were stably transfected into PC-3 cells. *A*, expression of RelA and RelB in the selected clones was measured by Western blots. β-Actin served as a loading control. *B*, after implantation of clones into the prostates of mice, plasma samples were collected to quantify IL-8. *C*, pathologic analysis of orthotopic transplant tumors. The morphology of all primary tumors was undifferentiated prostatic carcinoma. The sizes of primary prostate carcinomas in the control siRNA group were much bigger than in the RelB siRNA group.

Figure 4. Enhancement of LNCaP cell tumorigenicity through overexpression of RelB. A RelB expression plasmid and empty vector were stably transfected into LNCaP cells. A, total proteins were extracted from the selected clones and RelA and RelB were measured by Western blots. B, transformation capacities of the selected clones were determined by soft agar assay. C, selected clones were implanted into mice prostates and plasma samples were collected from the mice on indicated days. Concentrations of PSA and IL-8 in plasma samples were quantified. D, tumors were excised and protein levels of RelA, RelB, and target proteins were measured by Western blots. The results obtained from quantifying multiple Western blots for several tumor samples were plotted. β -Actin served as loading control.