Induction of neuronal apoptosis inhibitory protein expression in response to androgen deprivation in prostate cancer

Helen H.L. Chiu a, Theresa M.K. Yong a, Jun Wang a, Yuwei Wang b, Robert L. Vessella c, Takeshi Ueda d, Yu-Zhuo Wang b, Marianne D. Sadar a, *

a Canada’s Michael Smith Genome Sciences Centre, British Columbia Cancer Agency, 675 West 10th Avenue, Vancouver, British Columbia, Canada V5Z 1L3
b Department of Cancer Endocrinology, British Columbia Cancer Agency, 675 West 10th Avenue, Vancouver, British Columbia, Canada V5Z 1L3
c Department of Urology, University of Washington Medical Center, 1959 N.E. Pacific Street, Seattle, WA 98195, USA
d Division of Urology, 666-2 Nitona-cho, Chuo-ku, Chiba Cancer Center, Chiba 260-8717, Japan

Article info
Article history:
Received 15 October 2009
Received in revised form 30 November 2009
Accepted 30 November 2009
Keywords:
Prostate cancer
Androgen deprivation
Nuclear factor-κB
Inhibitors of apoptosis
Neuronal apoptosis inhibitory protein

Abstract
A mechanism for survival of prostate cancer cells in an androgen-deprived environment remains elusive. Here, we find that expression of neuronal apoptosis inhibitory protein (NAIP) was significantly increased in vivo and in vitro in response to androgen deprivation therapy (ADT). Increased expression of NAIP corresponded to increased DNA-binding activity of NF-κB that physically associated to previously uncharacterized κB-like sites in the NAIP locus. Importantly, expression of NAIP was significantly increased (p = 0.04) in clinical samples of prostate cancer from patients receiving ADT. Expression of NAIP may be associated with enhanced survival of prostate cancer in response to castration.

© 2009 Elsevier Ireland Ltd. All rights reserved.

1. Introduction
Androgen deprivation therapy (ADT) is an effective approach for the treatment of advanced prostate cancer (CaP). This therapy is based on the prostate’s dependency on androgen to grow and survive. Unfortunately, despite the initial responsiveness to ADT, the cancer will eventually recur and progress to castration-resistant disease. The molecular mechanisms by which CaP cells survive under androgen-depleted conditions are unknown. Enhanced expression of prosurvival proteins is suspected to render some cancer cells less prone to cell death induced by androgen deprivation.

A family of proteins termed inhibitors of apoptosis proteins (IAPs), characterized by the presence of one or more baculoviral IAP repeat domains, is capable of rescuing cells destined for death via the caspase cascade [1]. Eight human IAPs have been identified. These are the neuronal apoptosis inhibitory protein (NAIP/BIRC1), c-IAP1 (BIRC2/HIAP2), c-IAP2 (BIRC3/HIAP1), XIAP (BIRC4), survivin (BIRC5), apollon (BIRC6), livin (BIRC7/KIAP/ML-IAP) and IAP-like protein-2 (BIRC8/ILP-2) [2–8]. IAPs primarily function by restraining the activity of the caspase family. For example, NAIP directly inhibits the cell death effector proteases, caspase-3 and caspase-7 [9] and associates with the initiator caspase, caspase-9 [10]. Thus, it is not surprising that IAPs are deregulated in various malignancies (reviewed in [11]). Elevated expression of IAPs is an early event in the pathogenesis of CaP [12]. Emerging studies imply a role of IAPs in conferring drug-resistance in CaP cells [13–15]. However, the role of IAPs in response to ADT in CaP is understudied.

The cytoprotective properties of IAPs are associated with the nuclear factor (NF)-κB signalling pathway (reviewed by [11,16]). c-IAP1, c-IAP2, XIAP and survivin are NF-κB targets [17–20]. The ubiquitously expressed NF-κB family includes p65/RelA, RelB, c-Rel, p50/p105/NF-κB1 and p52/
p100/NF-κB2. Homodimers and heterodimers comprising these subunits regulate a multitude of genes and proteins that are involved in survival and programmed cell death among a variety of biological functions (reviewed in [21]). Although other non-canonical pathways have been described, the activation of NF-κB typically involves phosphorylation of the inhibitor of κB (IκB) by IκB kinase complex. Subsequent ubiquitlation and proteosomal degradation of IκB allows the nuclear translocation, DNA-binding and transcriptional regulation of NF-κB on target genes by binding to the κB sites in the gene loci. The NF-κB signalling pathway is associated with the development and progression of CaP and as well as other malignancies (reviewed in [22–24]). Constitutively active NF-κB, p65/p50 heterodimer, has been implicated in the resistance of CaP to apoptosis and disease progression [25–28]. NF-κB can inhibit apoptosis by transcriptional regulation of anti-apoptotic genes [29].

Here, we investigated the effect of androgen deprivation on IAPs and NF-κB in CaP. Since NAIP showed significantly increased expression in response to androgen deprivation, it was the focus of the study. Increased expression of NAIP in response to ADT suggests a potential mechanism by which some CaP cells may acquire enhanced survival.

2. Materials and methods

2.1. Animal models

The LNCaP hollow fiber model was performed in male athymic nude mice as described [30]. Serum PSA was measured using the IMx® Total PSA Assay (Abbott Laboratories). Mice were castrated 7 days after implantation of the fibers. A group of five mice were left intact from any major surgical procedure. Two additional groups of mice were included to control for the surgery. For these two groups of control mice, either a mock castration was performed by making the incision without removal of the testicles, or subcutaneous implantation of testosterone pellet (2.5 mg; Innovative Research of America, Sarasota, FL) upon castration. Subcutaneous LNCaP xenografts were prepared with Matrigel® in male NOD-SCID mice. Human CaP transplanted tumor lines, PCa1, AB163, AB220M, were derived from advanced CaP and maintained in male NOD-SCID mice [31]. Two pieces of $2 \times 2 \times 1 \text{ mm}^3$ tissue were grafted beneath the renal capsule of adult mice. A set route of administration, dosage and schedule were followed for docetaxel treatment (Sanofi-Aventis). Fourteen days after grafting, the mice were divided into two groups: one was administered with docetaxel by intraperitoneal injection (dose of 15 mg/kg) on days 14 and 17. Control mice were given saline as vehicle control. On day 20, mice were euthanized and grafts harvested and examined for genes by binding to the κB sites in the gene loci. The NF-κB signalling pathway is associated with the development and progression of CaP and as well as other malignancies (reviewed in [22–24]). Constitutively active NF-κB, p65/p50 heterodimer, has been implicated in the resistance of CaP to apoptosis and disease progression [25–28]. NF-κB can inhibit apoptosis by transcriptional regulation of anti-apoptotic genes [29].

Here, we investigated the effect of androgen deprivation on IAPs and NF-κB in CaP. Since NAIP showed significantly increased expression in response to androgen deprivation, it was the focus of the study. Increased expression of NAIP in response to ADT suggests a potential mechanism by which some CaP cells may acquire enhanced survival.

2.2. RNA extraction and RT-PCR

Total RNA was extracted from in vitro cells using Trizol® Reagent (Invitrogen). For xenografts, cubes of 2–5 mg of tumor tissue were homogenized and total RNA was isolated using the RNA Easy Micro Kit (Qiagen). Reverse transcription (RT-PCR) and real-time quantitative PCR (qPCR) were performed separately in triplicates for each biological sample. Poly(A)⁺ RNA was reverse transcribed using oligo(dT) and the SuperScript™ III First-Strand Synthesis System. qPCR was performed with Platinum® SYBR® Green qPCR SuperMix-UDG with ROX (Invitrogen). Primers were designed to generate PCR products of <200 bp (Suppl. Table 1). Results were reviewed using the SDS 2.2 software (Applied Biosystems). Levels of expression were normalized to levels of GAPDH mRNA to give the mean normalized expression (MNE). MNE = CT value of reference gene (primer efficiency)/CT value of target gene (primer efficiency).

2.3. Protein extracts and Western blot analyses

Protein extracts were prepared from cells lysed in hypotonic buffer [33]. The supernatant (cytosolic fraction) was removed and stored. The pellet was resuspended in high salt buffer to extract nuclear proteins. Protein concentration was quantified by Bradford assay (Bio-Rad). Western blot analysis of NAIP employed cytosolic extracts (60 μg protein) with anti-human NAIP antibody (ab25968, Abcam). The membranes were stripped with Restore™ Western Blot Stripping Buffer (Pierce) and reprobed with monoclonal anti-β-actin (ab8226, Abcam).

2.4. Electrophoretic mobility shift assay (EMSA)

Double-stranded oligonucleotides (22 base pairs), NF-κB consensus oligonucleotide (5’-AGTTGAGGGACTTTCCAGGC-3’), NF-κB mutant oligonucleotide (5’-AGTTGAGGGACTTTCCAGGC-3’), kB-like-1 consensus oligonucleotide (5’-ATTCAAGGGATTTACAGTCAT-3’), kB-like-1 mutant oligonucleotide (5’-ATTCAAGGGATTTACAGTCAT-3’), kB-like-2 consensus oligonucleotide (5’-AGGATGGGGCTATCCCCTGAA-3’), kB-like-2 mutant oligonucleotide (5’-AGGATGGGGCTATCCCCTGAA-3’), kB-like-3 consensus oligonucleotide (5’-ATAGAAGCTAATTTCCCAGGCT-3’), kB-like-3 mutant oligonucleotide (5’-ATAGAAGCTAATTTCCCAGGCT-3’) were radiolabelled and used for EMSA. Supershift assays preincubated nuclear extracts with anti-p65 antibody or anti-p50 antibody. Competition binding assays preincubated nuclear extracts with 250-fold excess unlabelled oligonucleotides. The density of the shifted band that corresponded to a protein-DNA complex was analyzed using ImageQuant® 5.2 (GE Healthcare).
2.5. In vitro androgen deprivation

LNCaP cells were incubated in RPMI 1640 supplemented with 5% charcoal-stripped bovine serum with 10nM DHT for 20 h. The cells were washed with serum-free medium (SFM) to remove residual DHT. For in vitro androgen deprivation, the media was replaced with fresh SFM and the cells cultured in the androgen-deprived environment. Control cells were maintained in SFM supplemented with 10nM DHT after washing. Cells were harvested after another 27 h of incubation.

2.6. NF-κB luciferase reporter activity assay

LNCaP cells were transfected with a NF-κB luciferase reporter vector (Panomics) containing six tandem copies of the consensus κB site using Lipofectin reagent (Invitrogen). After 24 h, cells were treated for an additional 24 h before harvesting and measurement of luciferase activity normalized to protein concentration.

2.7. Chromatin immunoprecipitation (ChIP)

After plating cells, the media was replaced with SFM for an additional 24 h. Cells were treated with SFM supplemented with BSA (1 mg/ml) in the presence or absence of TNF-α (10 ng/ml) for 30 min. The proteins were cross-linked with formaldehyde, washed, pelleted, and lysed. The cell extract was sonicated to give an average length of 200–800 bp of sheared DNA fragments. The antibody–protein–DNA complex was precipitated using qPCR experiments are shown in Suppl. Fig. 1A.

3.1. ADT increases levels of NAIP transcript in vivo

The LNCaP hollow fiber model restricts contamination of cells within the fiber by host cells [30] and was employed to investigate the levels of expression of the IAPs genes, NAIP, c-IAP1, XIAP, and survivin in response to ADT. Total RNA was isolated from LNCaP cells retrieved before castration (7 days after implantation) and 10 days after castration (17 days after implantation). The levels of mRNA for NAIP, c-IAP1, XIAP and survivin, were measured by qPCR (Fig. 1). Levels of PSA mRNA were decreased in response to castration as expected and consistent with clinical representation [34,35]. Levels of mRNA for c-IAP1, XIAP were not significantly changed in response to ADT at this time point. However, both survivin (decreased, p < 0.01) and NAIP (increased, p < 0.01) showed significant changes in levels of mRNA in response to ADT.

3.2. ADT increases NF-κB activity

NF-κB may promote anti-apoptotic properties via transcriptional regulation of survival genes such as c-IAP2 [36] and survivin [17]. This prompted us to explore levels and biological activity of NF-κB in CaP in response to ADT using in vivo samples. Serum PSA levels dropped by an average of 57% by 10 days after castration of mice implanted with fibers containing LNCaP cells (Fig. 2A). Nuclear levels of NF-κB (p65 and p50) proteins were unchanged in extracts obtained from the LNCaP hollow fiber model before and after castration as measured by western blot analysis (Fig. 2B). Quantification of protein bands obtained from the replicate experiments are shown in Suppl. Fig. 1A.

To determine the effect of ADT on the DNA-binding activity of NF-κB, aliquots of nuclear extracts (20 μg protein in each lane) prepared from the LNCaP hollow fiber model before castration and after castration were analyzed using EMSA (Fig 2C). Nuclear extracts from cells treated with tumor necrosis factor-α (TNF-α) were included as a positive control and showed strong bands corresponding to p65/p50 and p50/p50 complex formation with DNA (compare lanes 2 and 3). The specificity of the shifted bands corresponding to the different NF-κB DNA complexes was confirmed by supershift assay in the presence of anti-p65 or anti-p50 antibody (lanes receiving ADT were obtained from the Prostate Cancer Rapid Autopsy Program at the University of Washington through co-author RLV. Informed consent was obtained from each patient in compliance with ethical and scientific standards (UBC/BCCA Research Ethics Board).

3. Results

3.1. ADT increases levels of NAIP transcript in vivo

Fig. 1. Levels of IAP mRNA in response to ADT. Levels of mRNA for each gene was normalized to the mRNA levels of GAPDH in each biological replicate obtained from the LNCaP hollow fiber model before (pre-Cx) and 10 days after castration (post-Cx). Bars = mean fold-change relative to the pre-castrate levels (1-fold) from three different animals with technical triplicates for each animal. 2-way ANOVA: *, p < 0.05, **, p < 0.01.
Fig. 2. Expression and DNA-binding activity of NF-κB in response to ADT. (A) Serum PSA levels in response to castration were normalized to the number of hollow fibers remaining in the hosts. Pre-castrate levels of PSA were in the range of 14–18 ng/ml and set at 100% for each host. The bars represent the mean percentages of the pre-castrate serum PSA levels ±SD (n = 4) before (pre-Cx) and 10 days after castration (post-Cx). (B) Levels of p65, p50, and β-actin (loading control) proteins in nuclear extracts from cells harvested from the hollow fiber model. Solid bars mark protein extracts from the same animal (n = 3). (C) EMSA using nuclear extracts (5 µg protein) obtained from the hollow fiber model from pre-Cx (lane 8) and post-Cx (lane 9) with consensus NF-κB DNA-binding motif. Controls include: BSA (lane 1), nuclear extracts of untreated (lane 2) and TNF-α-stimulated cells (lane 3) that were preincubated with antibodies (Ab) for p65 (lane 4) or p50 (lane 5) or an excess of non-labelled consensus (cNF-κB, lane 6) or mutant (mNF-κB, lane 7) NF-κB oligonucleotides. SS: supershifted antibody-protein-DNA complex. The bands corresponding to the NF-κB (p65/p50)–DNA complex in EMSAs performed using matched samples (pre-Cx and post-Cx) from three different animals were quantified using densitometry and presented in bar graph. Solid bars mean fold-change of the biological triplicates of post-Cx compared with the pre-Cx levels (set as 1-fold). (D) Serum PSA levels, NF-κB expression, and DNA-binding activity were assessed in LNCaP cells obtained from the procedural control mice (n = 4 for each set of controls) on 7 days and 17 days after implantation. Intact, no surgical procedure was performed on the mice throughout the experiment; mock Cx, a small incision in the scrotum was made without removal of the testicles 7 days after implantation; Cx + T, a testosterone pellet was added to each mouse upon castration 7 days after implantation of fibers. All images are representative of biological replicates. 2-way ANOVA: *, p ≤ 0.05; **, p ≤ 0.01.
4 and 5) or competition assay in the presence of excess, non-labelled, consensus (lane 6) or mutant oligonucleotide probes (lane 7). The band corresponding to p65/p50 in lane 2 was shifted with an antibody to p65 (lane 4), although the new band formed by the antibody–NF-κB DNA complex was weak (upper “SS”) which may reflect reduced affinity that can occur with DNA-binding complexes. NF-κB DNA-binding activity was elevated substantially in response to castration (compare lane 9 to lane 8). The densities of the bands corresponding to the NF-κB (p65/p50)–DNA complexes were quantified and the average fold-induction was over 2.5-fold relative to the pre-castrate levels from biological triplicates (Fig. 2C).

Three groups of procedural control mice [i.e. intact, mock castration (Cx), castration with the addition of testosterone pellet (Cx + T)] were maintained throughout the same length of time as the hollow fiber model experiment with castrated mice. Likewise with mice subjected to castration, cells were collected at the relative times to assess for the expression and DNA-binding activity of NF-κB. The results are displayed in parallel with the data from mice subjected to castration (Fig. 2D). No significant change in serum PSA levels in intact mice and mock Cx mice was observed in 7 days and 17 days after implantation of fibers except for Cx + T mice. Nuclear extracts prepared from samples obtained at 7 days and 17 days after implantation from intact animals had similar NF-κB expression and DNA-binding activity (Suppl. Fig. 1B). This suggests that the length of time in the fiber had no appreciable effect on NF-κB DNA-binding activity. Similarly, nuclear extracts prepared from samples obtained from mock-castrated animals, or castrated mice supplemented with testosterone also had no difference in NF-κB expression and DNA-binding activity (Suppl. Fig. 1B). Thus, the results from these in vivo procedural controls confirm that the results obtained from the castrated mice were not due to artifacts from the invasive surgical procedures performed on the hosts.

### 3.3. Androgen alters transcriptional activity of NF-κB and expression of NAIP

In the presence of androgen, there is crosstalk between androgen receptor (AR) and NF-κB [37,38]. To test if androgen had a direct effect on NF-κB activity in our model, the transcriptional activity of NF-κB was evaluated using a luciferase reporter gene construct containing six κB sites. Treatment of LNCaP cells, that express AR, with TNF-α (positive control) strongly induced the activity of this reporter indicating increased NF-κB transcriptional activity (Fig. 3A). Treatment of LNCaP cells with synthetic androgen R1881 significantly decreased NF-κB activity as compared to the vehicle control. Thus, androgen inhibited the transcriptional activity of NF-κB.

To test whether the in vivo increased levels of NAIP mRNA were due to reduced androgen, total RNA was isolated from androgen deprived LNCaP cells maintained in vitro. The expression of PSA (control for androgen deprivation) and NAIP were evaluated by qPCR. Levels of PSA mRNA were significantly decreased, as expected, while levels of NAIP mRNA were significantly increased (p < 0.01) in the cells subjected to androgen deprivation (Fig. 3B).

Consistent with increased levels of NAIP mRNA in response to ADT, levels of NAIP protein was also increased with castration as compared to levels in xenografts from non-castrated hosts (Fig. 3C). The increased levels of NAIP protein was localized near the periphery of the tumor tissue near blood vessels. The heterogeneity of NAIP staining could possibly be attributed to macrophages residing in these tissues [39]. Therefore, levels of NAIP protein were also measured by Western blot analysis using extracts of LNCaP cells harvested from the hollow fiber model that restricted infiltration of host cells. Levels of NAIP protein, at approximately 160-kDa, were consistently elevated in cells obtained from animal hosts (n = 3) subsequent to castration as compared to levels from non-castrated hosts (Fig. 3D). The increased levels of NAIP protein were consistent with the increased levels of NAIP transcript in CaP cells in response to ADT. Together, the data suggest that ADT increases the expression of NAIP in CaP.

### 3.4. Recruitment of NF-κB to the NAIP locus

Increased NAIP expression followed the same trend observed for NF-κB DNA-binding activity in response to androgen deprivation and suggests a possible association between NAIP and NF-κB. Consistent with this observation, there has been speculation by others that the expression of NAIP may be directly regulated by NF-κB [40,41]. However biological evidence has yet to be provided. The human NAIP locus [GeneBank Accession No. U19251] [42] was therefore examined for putative κB sites (CCGRNNYYCC [R = purine, N = any base, Y = pyrimidine]) using ConSite [http://aspi.uib.no:8090/cgi-bin/CONSITE/consite] [43] with an 80% cutoff. Initial screening with ConSite revealed two κB-like sites in the promoter region and one κB-like site within the second intron of NAIP that were highly homologous to the consensus κB site (Table 1). To test NF-κB binding on these κB-like sequences, EMSA was employed using
custom oligonucleotide probes that contain the kB-like sites and nuclear extracts from in vitro and in vivo (i.e. from the hollow fiber model) LNCaP cells. Supershift and competition assays were performed to confirm the specificity of the NF-kB-DNA complexes. Nuclear extracts from cells treated with TNF-α were used as a robust positive control and produced NF-kB complexes on all three kB-like sites (Fig. 4A). NF-kB DNA-binding activity in nuclear extracts from in vivo samples demonstrated enhanced DNA-binding activity in samples from castrated hosts on all kB-like sites with kB-like site-3 > kB-like site-1 > kB-like site-2. NF-kB complexes binding on these kB-like sites suggested that the expression of NAIP may be transcriptionally regulated by NF-kB binding to these sites located within the promoter and intronic regions in the gene locus.

The physiological relevance of NF-kB binding on these kB-like sites in the promoter and intronic regions of NAIP, was validated by ChIP assays in cells stimulated with TNF-α. Subsequent to cross-linking of protein-DNA complexes, immunoprecipitations were performed using an antibody specific for p65. Primers specific for the kB-like sites on the promoter and intronic regions of NAIP were used to amplify the immunoprecipitated DNA using qPCR. Immunoprecipitation of sonicated nuclear extracts with rabbit IgG was performed in place of anti-p65 antibody as controls for no antibody. The recruitment of p65 was enhanced on all kB-like sites in the NAIP locus in response to TNF-α as compared to the recruitment in the vehicle control (Fig. 4B). Recruitment on kB-like-3 site that is located within the second intronic region demonstrated statistically significant increase in physical association by ChIP which was consistent with the 1.9-fold increase in DNA-binding activity on kB-like-3 site obtained using EMSA (p < 0.05) (Suppl. Fig. 2).

3.5. Elevated NAIP expression in chemoresistant tumors with reduced apoptosis

Docetaxel is an effective chemotherapy agent approved for castration-resistant CaP [44]. A transplantaible xenograft model originally derived from human CaP [31] was employed to test whether endogenous levels of NAIP expression correlated to response to docetaxel and apoptosis. Each of these transplantable xenografts had 100% take rate and similar doubling times of 3–4 days. Animals were treated with docetaxel as well as control injection of carrier (i.e. saline). Total RNA was extracted from the xenografts and subjected to qPCR. The level of NAIP mRNA was significantly higher in PcA1 xenografts than AB163 or AB220M xenografts regardless of exposure to docetaxel (Fig. 5A). To test the susceptibility to docetaxel treatment in these different xenografts, tumor volume in control and treated mice was evaluated at the endpoint. The tumor volume of PCa1 was not significantly altered, while AB163 and AB220M with low levels of endogenous NAIP, regressed significantly in response to docetaxel (Fig. 5B). These transplantable tumor lines inately show a different responsiveness to docetaxel. The efficacy of docetaxel in different xenografts was compared by treatment to control (T/C value) calculation. Comparing with the T/C value, the efficacy of docetaxel was the least in PCa1 (53.6%) followed by AB220M (33.9%) and AB163 (5.48%). Both AB163 and AB220M xenografts can be assessed as sensitive lines to docetaxel, since the criterion for efficiency of the T/C ratio is <42% according to National Cancer Institute standards [32]. To further examine the resistance to apoptosis in these xenografts in response to docetaxel, the TUNEL assay was employed to measure the apoptotic cells in these tumors. PCa1 xenografts were not significantly susceptible to docetaxel as compared to AB163 and AB220M which demonstrated significant increase in apoptosis (Fig. 5C). TUNEL staining images highlight the contrast in number of apoptotic cells observed in PCa1 versus

<table>
<thead>
<tr>
<th>Site</th>
<th>Coordinates</th>
<th>Sequence</th>
<th>Identity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Consensus</td>
<td>^GGGRNYYYYYCC</td>
<td>10 nucleotides of the NF-κB-like sequence.</td>
<td></td>
</tr>
<tr>
<td>kB-like-1</td>
<td>1520 to 1511</td>
<td>GGGAATTAC</td>
<td>9/10</td>
</tr>
<tr>
<td>kB-like-2</td>
<td>241 to 232</td>
<td>GGCGTATCC</td>
<td>9/10</td>
</tr>
<tr>
<td>kB-like-3</td>
<td>498–507 bp 5’ to exon 3’</td>
<td>GGAATTTAC</td>
<td>9/10</td>
</tr>
</tbody>
</table>

* Coordinates determined from the sequence are based on GeneBank Accession No. U19251 [42].

\( R = \text{any purine, } N = \text{any nucleotide, } Y = \text{any pyrimidine.} \\
\( ^* \text{Identity indicates the number of nucleotides which are identical to the 10 nucleotides of the NF-κB consensus sequence.} \\

\( ^\text{1} \text{Coordinates determined from the sequence are based on GeneBank Accession No. U19251 [42].} \\

\( ^\text{2} \text{R = any purine, } N = \text{any nucleotide, } Y = \text{any pyrimidine.} \\

\( ^\text{3} \text{Identity indicates the number of nucleotides which are identical to the 10 nucleotides of the NF-κB consensus sequence.} \\

\( ^\text{4} \text{Coordinates determined from the sequence are based on GeneBank Accession No. U19251 [42].} \\

\( ^\text{5} \text{R = any purine, } N = \text{any nucleotide, } Y = \text{any pyrimidine.} \\

\( ^\text{6} \text{Identity indicates the number of nucleotides which are identical to the 10 nucleotides of the NF-κB consensus sequence.}
AB163 or AB220M (Fig. 5D). Results from this transplantable CaP xenograft model showed that expression of NAIP is positively correlated with the inhibition of both tumor regression and apoptosis in response to docetaxel.

3.6. Expression of NAIP in clinical samples

Levels of PSA and NAIP mRNAs were examined in CaP from men who were treated with or without ADT. Tissue levels of PSA mRNA was significantly reduced in patients (n = 12) that received ADT (p = 0.0056) (Fig. 6A), while levels of NAIP mRNA in these same samples were significantly elevated (p = 0.0403) as compared to the levels measured in patients (n = 6) without ADT (Fig. 6B). These data are consistent with both in vitro and in vivo results that ADT increases expression of NAIP.

4. Discussion

Progression of CaP to castration-resistance may involve outgrowth of CaP cells in response to ADT with distinct molecular properties that are resistant to apoptosis. Accumulating evidence support the role of IAPs in CaP as anti-apoptotic regulators (reviewed in [45]). Here we investigated expression of NAIP in CaP in response to ADT and revealed the following: (1) levels of NAIP mRNA and protein increased in vivo in response to ADT; (2) NF-κB DNA-binding and transcriptional activities increased in response ADT; (3) NF-κB binds to two κB-like sites in the promoter region and one κB-like site within the second intron of the NAIP locus; (4) high levels of endogenous expression of NAIP correlated to resistance to apoptosis by docetaxel in CaP xenografts; and (5) clinical samples of CaP with ADT have significantly higher expression of NAIP (p = 0.04) compared to CaP with no ADT.

Examination of changes in expression of several of the IAP genes revealed that NAIP was significantly increased in response to ADT in vivo and in clinical CaP specimens.
from men treated with ADT. The expression of survivin was significantly decreased, while the expression of c-IAP1 and XIAP were not significantly altered in the hollow fiber model. Survivin is predominantly expressed in the G2/M phase of the cell cycle and promotes mitosis in rapidly dividing cells (reviewed in [46]). ADT initially results in decreased proliferation in LNCaP hollow fiber model [30]. Decreased mRNA levels of survivin in this in vivo model in response to castration of the hosts may reflect reduced proliferation. Consistent with the data presented here, down-regulation of survivin was reported in LNCaP cells maintained in androgen-deprived conditions in vitro [47].

Survivin, c-IAP1 and XIAP are all transcriptionally regulated by NF-κB [18,19] and here we provide evidence that the NAIP locus contains functional NF-κB binding sites. However, we did not observe significant increases in expression of all of these genes at 10 days after castration in spite of the increased NF-κB DNA-binding activity. These gene-specific differences may be due to the subtle changes in NF-κB activity demonstrated at the designated time in response to ADT or differences in the kinetics of individual genes. The expression of these genes might require different factor(s) which facilitate the NF-κB signalling and the transcriptional regulation may vary depending on the cellular context and experimental conditions. Collecting in vivo samples from additional times after castration may help to address these unknowns by providing a comprehensive profile of NF-κB activity and IAPs expression in response to ADT.

NAIP is the founding member of the family of human IAPs. Deficiency of NAIP from deletions of a gene region is primarily associated with the most severe phenotypes of a hereditary neurodegenerative disorder, spinal muscular atrophy (SMA), due to loss of its neuroprotective activity in motor neurons in the spinal cord [8]. Expression of NAIP in tissues that are not exclusively neuronal and not directly associated with SMA suggests functions beyond its neuronal context [39,48]. NAIP protects mammalian cells from apoptosis induced by a variety of stimuli [6]. Although no study has been specifically performed in cancer cells, extrapolating the function of NAIP in benign cells would strongly suggest NAIP would also protect cancer cells from apoptosis. Consistent with this role, we observed a correlation of overall sensitivity and reduced apoptosis in response to docetaxel in CaP tissue that expresses elevated levels of NAIP. The absence of strict correlation of sensitivity to docetaxel in tumor lines AB163 and AB220M, that had extremely low levels of NAIP mRNA compared to PCa1, may be attributed to the contribution of other molecular mechanisms when NAIP is poorly expressed. The presence of a central nucleotide binding oligomerization domain and a carboxyl-terminal leucine-rich repeat domain might enable NAIP to promote additional cytoprotection and other functions unique from other IAP members [10]. However, the events regulating NAIP are largely unknown.

To begin to address this void, we observed for the first time a link between NF-κB activity and NAIP expression. ADT increased levels of NAIP mRNA and NF-κB DNA-binding activity, and the complementary experiment showed that the presence of androgen inhibited NF-κB activity. The reduction in NF-κB transcriptional activity in the presence of androgen as demonstrated by the NF-κB luciferase reporter assay suggests that the differential NF-κB DNA-binding activity evident from in vivo LNCaP cells corresponded to the androgen levels in the microenvironment of the CaP cells. This means that when a host bearing CaP cells is castrated, an increase in NF-κB DNA-binding activity should be observed. Curiously, nuclear levels of NF-κB did not correlate to its binding activity in extracts prepared from samples maintained in vivo in response to castration of the hosts. Thus, NF-κB activity in vivo may be modulated by post-translational modification of the NF-κB subunits, such as phosphorylation and acetylation status of the NF-κB subunits (reviewed in [49]). In cell culture, crosstalk between AR and p65 have been shown to mutually repress the transactivation activity of each protein [37,38]. Consistent with those studies, here transactivation of a NF-κB luciferase reporter construct yielded comparable inhibition in the presence of androgen similar to that used previously [38] in LNCaP cells with endogenous AR.

Elevated levels of NAIP that correlated with increased NF-κB binding activity shown here are consistent with decreased levels of NAIP mRNA in human hepatic cancer cells treated with dehydroxymethylepoxyquinomicin, an NF-κB inhibitor [40] and increased NAIP expression in leukemia cells with constitutive activation of NF-κB [41]. Together these data support that NAIP may be transcriptionally regulated by NF-κB. Here, the application of EMSA reveals NF-κB DNA-binding to three previously uncharacterized NF-κB regulatory binding elements in the NAIP promoter and intronic regions. ChIP experiments validated significantly increased NF-κB interaction in situ on the regulatory element in the second intron. Although these ChIP experiments did not show statistically significant increases in NF-κB interaction on the other κB-like sites in the promoter region, they may still be functional κB-like sites with possibly lower affinity requiring further optimization with a different set of experimental conditions. NAIP expression may be regulated by multiple factors including PAX2 [50], a developmental transcription factor and Brn-2 [51], a POU domain transcription factor. However, the binding of PAX2 and Brn-2 to their respective putative reg-
ulatory elements on the NAIP locus was only demonstrated by EMSA, an in vitro assay. Thus, the binding in situ has yet to be confirmed using methods such as ChIP as used here. Differences in cofactors involved in the transcriptional complexes under different cellular conditions may be important for regulation of expression of NAIP and putative regulatory elements of other transcription factors are yet to be validated [51]. Our findings demonstrate that NF-κB can regulate the transcription of the NAIP gene through cis-regulatory elements that resemble the NF-κB consensus binding motif. Intriguingly, the κB-like-3 site which demonstrated the significant increase in binding upon TNF-α stimulation lies 5′ upstream and in close proximity to the constitutive transcription start site within the non-long-terminal-repeat promoter [48]. The resulting transcript would yield the same protein product as the commonly-cited transcript [42].

In summary, this first report on investigation of NAIP in CaP reveals elevated expression of NAIP in response to ADT and a link between NF-κB activity and expression of NAIP potentially involving functional NF-κB binding sites in the promoter and intronic regions of the NAIP gene. The clinical relevance of elevated expression of NAIP is supported by the profile expression of NAIP in CaP patients with ADT. Together with observations from other groups, the cumulative data suggest future studies that entail deciphering the specific function of NAIP in CaP progression to castration-resistant disease and prognostic value to predict response to chemotherapy are warranted.

Conflicts of interest

The authors declare that they have no competing interests.

Acknowledgements

We thank Nasrin R. Mawji, Rebecca Wu, Gang Wang and Tammy Romanuik for their technical assistance. This research was supported by funding from NIH R01 CA105304 (to M.D.S.).

Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.canlet.2009.11.023.

References


