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Advanced prostate cancer continues to kill 29,000 men annually in the United States. Despite strides in obtaining extended remissions, there is still no curative therapy for this devastating disease. While most prostate cancers are responsive to androgens and while androgen withdrawal is the main form of treatment, the failure of primary hormone therapy is attributed to androgen-independent tumor expansion. Mechanisms for the transition from androgen-sensitive to androgen-refractory disease are currently not well understood. We proposed to investigate the role of TNF-α-mediated NF-κB signaling in androgen-sensitive and -insensitive prostate cancer, as many androgen-independent prostate cancer cell lines exhibit resistance to this pathway and as TNF-α is detected at high serum levels in relapsing prostate cancer patients compared to those in remission or untreated. During this current funding period we experienced difficulties in engineering dual luciferase and PSA-IκBα "super repressor" expressing cell lines for proposed *in vivo* experiments. We report characterization of PAR1 as a novel activator of NF-κB signaling and generation of an androgen-insensitive cell line from androgen receptor wildtype, androgen-sensitive VCaP. With recent access to new transfection technology, we anticipate swift creation of necessary sublines and will be poised to evaluate these novel insights of androgen interactions *in vivo*.

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## INTRODUCTION

Advanced prostate cancer continues to kill 29,000 men per year in the United States (Jemal *et al.*, 2004). Despite strides in obtaining extended remissions in men with metastatic disease through the use of hormones and chemotherapeutic agents, there is still no curative therapy. While most prostate cancers are responsive to androgens and while androgen withdrawal (i.e., surgical or medical castration) is the main form of treatment for advanced (i.e. disseminated) disease, the failure of primary hormone therapy is attributed to androgen-independent tumor expansion (Pilat *et al.*, 1999). The mechanisms for the transition from androgen-sensitive to androgen-refractory disease are currently not well understood but include the development of alternative signaling pathways to circumnavigate the effects of androgen ablation, leading to reactivation of androgen-responsive genes and disease progression (reviewed in Feldman and Feldman, 2001).

A complete understanding of all culprit proteins is essential for the effective translation of molecular reagents into successful tools for the medical management of prostate cancer. Toward this end, we were intrigued by observations that many androgen-independent prostate cancer cell lines exhibit a resistance to apoptotic signaling through the apoptotic factor, tumor necrosis factor, alpha (TNF-α; Nakajima *et al.*, 1996). In addition, TNF-α is detected at high serum levels in relapsing prostate cancer patients compared to those in remission or untreated (Nakashima *et al.*, 1998). In normal cells, TNF-α initiates either apoptotic or proliferation signaling pathways depending on its cellular concentration (reviewed in Gaur and Aggarwal, 2003). At high TNF-α levels, TNF receptor is bound, and the apoptotic caspase cascade is induced. However, at low levels, TNF-α uncouples the transcription factor NF-κB cytoplasmic inhibitor IκBα. The newly released NF-κB, a p50/p65 heterodimer, translocates to the nucleus, binds cAMP response element binding protein (CREB)-binding protein (CBP), and induces the transcription of anti-apoptotic genes. In addition to NF-κB, the transcriptional co-regulator CBP binds other sequence-specific factors including the androgen receptor (AR; Gerritsen *et al.*, 1997) as well as different DNA binding proteins and components of the general transcription machinery (Aarnisalo *et al.*, 1998). Both NF-κB and AR require CBP for their transactivations, although CBP has a greater affinity for NF-κB than AR (Aarnisalo *et al.*, 1998). In addition, as demonstrated in our preliminary data, AR is inactive when NF-κB is active. Finally, NF-κB has been implicated in the negative regulation of the rat AR gene promoter (Supakar *et al.*, 1995). Thus, the purpose of this project is to investigate the role of TNF-α-mediated NF-κB signaling in androgen-sensitive and -insensitive prostate cancer cells.

## BODY

As proposed in our approved Statement of Work, initial efforts on this project focused on engineering the prostate cancer cell lines LNCaP (PSA-producing; androgen-sensitive (AS)), C4-2B, a bone-derived subline of LNCaP (Thalmann *et al.*, 2000; PSA-producing; androgen-insensitive (AI)), and PC-3 (non-PSA-producing; (AI)) to express stably a PSA-IκBα "super repressor" construct. We achieved Specific Aim 1 and described in our 2002 Annual Report that by transfecting cells with the IκBα super repressor, TNF-α-induced apoptosis was activated (Muenchen *et al.*, 2000a), and NF-κB repression of the AR was relieved in AI prostate cancer cell lines (Muenchen et al, 2001a). We also showed that AS cells activate a different caspase pathway than AI cells (Muenchen *et al.*, 2001b), suggesting that we may be able to develop better therapeutic strategies for different populations of cancer cells (Muenchen *et al.*, 2000b;

Williams *et al.*, 2000). Finally, we demonstrated that stromal and cytokine components of the tumor microenvironment mediate androgen sensitivity and cancer cell behavior (Cooper *et al.*, 2002). Thus, our first year work generated a number of novel insights into androgen interactions in hormone-sensitive and hormone-refractory prostate cancer cells which have been more recently supported by other researchers (Andela *et al.*, 2003).

During our second year of funding year, we focused on Specific Aim 2 to generate an animal model to validate our *in vitro* results, a critical step toward advancing any agent as a potential novel therapy in the clinic. As spontaneous prostate cancer rarely occurs naturally in standard laboratory animals, metastases must be experimentally induced, most commonly by seeding immortalized human cancer cell lines into immunocompromised mice (Rosol *et al.*, 2003). More recently, these xenograft models have been improved by tagging cell lines, thereby distinguishing tumor cells from mouse cells (Edinger *et al.*, 2002). Using standard retroviral infection protocols, we marked the androgen- and TNF-α-insensitive prostate cancer cell line PC-3 with the bioluminescent-catalyzing enzyme luciferase ($PC\text{-}3^{Luc}$). Luciferase, when exposed to its substrate luciferin and in the presence of ATP generates light in a spectral range that readily transmits through living tissue (Rice *et al.*, 2001). Using a CCD camera the same animal cohort could be repeatedly reimaged over the course of an experiment, and thus the same tumors followed unlike other models requiring weekly sacrificing from large initial experimental groups. In our 2003 Annual Summary, we described generation of a sensitive, non-invasive murine model that used luciferase as an optical reporter for tumor location and that mimiced clinically observed tumors in early to late state metastatic prostate cancer (Kalikin *et al.*, 2003). Furthermore, we demonstrated the ability to assess empirically tumor growth and disease progression rates.

Thus, during this last year of funding we planned to apply the non-invasive luciferase model to evaluate expression of the PSA-IκBα super repressor construct *in vivo* with a focus on androgen receptor signaling. Unexpectedly, creation of dual-expressing luciferase and repressor stable sublines has been frustratingly unsuccessful. In particular, LNCaP and C42B are notorious for loose adherence to plastic and poor recovery after passaging and other manipulations. Multiple attempts have failed to generate sublines using standard cationic liposome transfection reagents. However, recently a collaborating UMichigan lab has purchased an Amaxa Nucleofactor II Instrument. Using state-of-the-art electroporation technology, transfection efficiencies are reported to be 50% or more in many cell lines, unprecedented efficiencies for other standard transfection protocols, and to work on many notoriously difficult-to-transfect lines. As well, Amaxa Biosystems have worked out transfection conditions for PC-3, LNCaP and Du145 so we are confidently optimistic that we will move quickly toward generating these subclones using this new technology.

While progress on the *in vivo* analysis of this grant was unexpectedly slow over the past year, we continued to make discoveries toward understanding better the role(s) of NF-κB signaling in prostate cancer biology. We recently discovered the G protein-coupled protease-activated receptor 1 (PAR1) as a novel activator of NF-κB. Previously we reported *PAR1* overexpression by microarray analysis in VCaP, a vertebral-derived prostate cancer cell line, compared to DuCaP, a dura-derived prostate cancer cell line (Chay *et al.*, 2002). Both cell lines were established in our laboratory from the same prostate cancer patient (Korenchuk *et al.*, 2001; Lee

*et al.*, 2001) through the rapid autopsy program at The University of Michigan (Rubin *et al.*, 2000). Further analysis showed *PAR1* overexpressed in other bone-derived prostate cancer cell lines compared to soft tissue-derived prostate cancer cell lines (see Fig. 1). In addition, PAR1 overexpression has been reported in other cancer types proportional to the degree of invasiveness (D'Andrea *et al.*, 2001; Liu *et al.*, 2001).

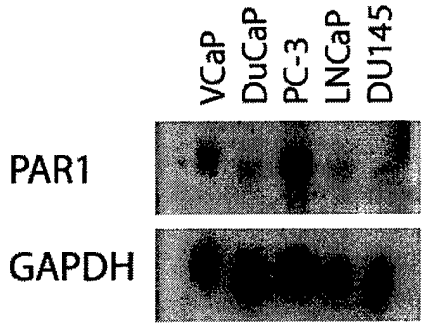



**Figure 1.** *PAR1* is overexpressed at the RNA level in bone-derived prostate cancer cell lines. This representative autoradiograph shows increased intensities of *PAR1* transcript in VCaP and PC-3 compared to other soft tissue-derived prostate cancer cell lines. A GAPDH probe was rehybridized to the same Northern blot to confirm equal loading of RNAs.

PAR1 is activated by thrombin, a multifunctional serine protease and key stimulator of the coagulation cascade, through irreversible cleavage at the receptor's extracellular amino terminus. This action unmasks a new tethered ligand which then serves as an intramolecular activator (Vu *et al.*, 1991). After 30 minute incubation of PC-3 cells with thrombin, activation of NF-κB was demonstrated by increased nuclear DNA binding activity of the p65 but not the p50 subunit at both the lower physiological (2 nM) and higher pharmacological (10 nM) concentrations of thrombin (see Fig. 2). As thrombin also activates other PAR family members, PAR1-specific induction was confirmed using equivalent concentrations of the synthetic PAR1-specific activator thrombin receptor activated peptide (TRAP6).

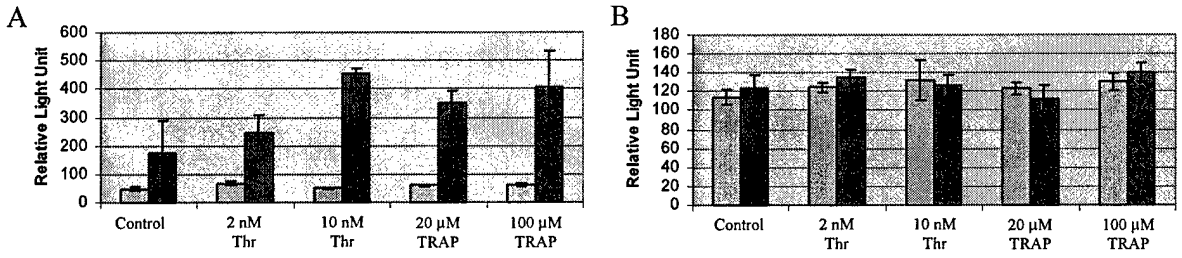



**Figure 2.** PAR1 activation increases DNA binding activity of nuclear p65 NF-κB subunit in PC-3 cells. Using a biotinylated oligonucleotide binding assay followed by ELISA detection with p65 (**A**) or p50 (**B**) antibody, cytoplasmic (▩) and nuclear fractions (■) were measured for colorometric detection of HRP-conjugated secondary antibody. Cells were serum starved for 2 hrs before 30 min exposure to thrombin (Thr) or TRAP6 (TRAP) in serum free medium. Results are expressed as mean ± standard deviation of triplicate repeats.

In Du145 cells, a dura-derived prostate cancer cell line, treatment with thrombin and TRAP6 to stimulate PAR1 showed similar results. Increased phosphorylation of nuclear p65 NF-κB subunit and cytoplasmic IκBα was also detected in thrombin and TRAP6 treated DU145 cells (see Fig. 3). As phosphorylation of IκBα is necessary to release its hold on the heterodimer p50/p65 NF-κB for translocation to the nucleus and as phosphorylation of p65 occurs concurrent to this, these data further demonstrated induction of the NF-κB pathway in response to PAR1 activation.

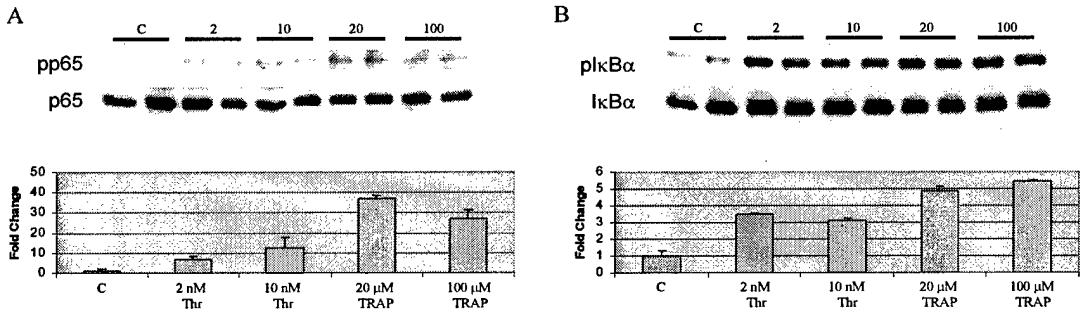



**Figure 3.** Stimulation of PAR1 in Du145 cells leads to increased phosphorylation of nuclear p65 and cytoplasmic IκBα proteins. Autoradiographs of Western hybridizations with anti-phosphorylated p65 (pp65) and anti-phosphorylated IκBα (pIκBα) are shown in the upper panel of (**A**) and (**B**) respectively. Antibodies detecting total p65 and IκBα were rehybridized to the same blots to confirm equal loading of lanes. Cells were serum started for 2 hrs followed by 30 min exposure to 2 nM (2) thrombin, 10 nM (10) thrombin, 20 μM TRAP6 (20), 100 μM TRAP6 (100), or no treatment (C), followed by nuclear (**A**) or cytoplasmic (**B**) protein isolation. In the lower portion of both panels densitometry was determined on autoradiographic bands, and the ratio of phosphorylated to total protein was calculated. Fold Change (Y axis) represents densitometry ratios from treated samples normalized to the untreated ratio with control (C) set at '1'. Results are expressed as mean ± standard deviation of duplicate repeats.

Furthermore, increased expression of IL-6, an NF-κB-induced cytokine heavily implicated for roles in prostate cancer pathogenesis and the development of AI disease (Park *et al.*, 2003 and references within), with PAR1 stimulation was detected (see Fig. 4). Interestingly, while PC-3 cells showed greater baseline expression of IL-6 than Du145, Du145 showed a higher fold increase in IL-6 expression affected by the PAR1-specific activator TRAP compared in PC-3. In addition, IL-6 production in PC-3showed increased stimulation in response to thrombin treatment compared to TRAP6 treatment, suggesting that other thrombin-responsive PAR family members such as PAR3 and PAR4 may induce IL-6 in some prostate cancers, although not necessarily through NF-κB.

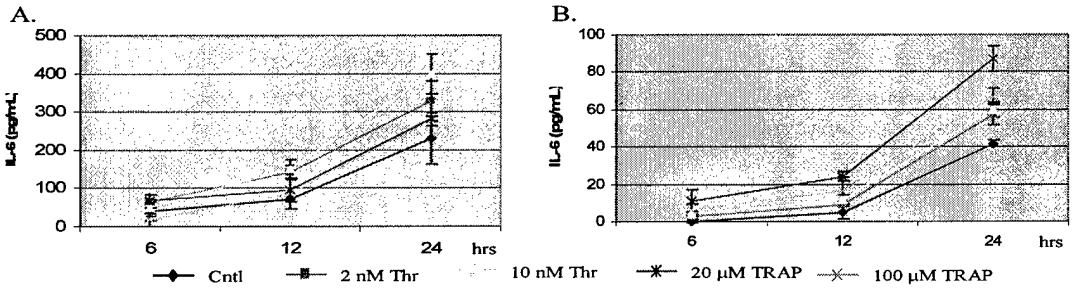



**Figure 4.** IL-6 expression is induced in prostate cancer cells in response to activation of the PAR1 signaling pathway. PC-3 (**A**) and Du145 (**B**) cells were serum starved for 24 hrs followed by thrombin (Thr) or TRAP6 (TRAP) treatment. Conditioned media was collected at the indicated time points (X axis) and IL-6 was quantitated by ELISA. Results are expressed as mean ± standard deviation of triplicate repeats.

In addition to addressing issues with gene transfer efficiency and to continuing to expand our understanding of NF-κB signaling, we also spent this past year further investigating AR signaling and the clinical transition from AS to AI disease. Toward this end, we began to generate AI sublines of VCaP and DuCaP as they are reportedly the only spontaneously immortalized prostate cancer cell lines that grow both *in vitro* and *in vivo* for which AR is wild type.

Subcutaneous VCaP and DuCaP tumors were generated in intact male immunocompromised (SCID) mice. Animals were humanely euthanized when tumors reached approximately 1 cM in diameter, tumors were harvested under sterile conditions, and new intact male SCID mice were

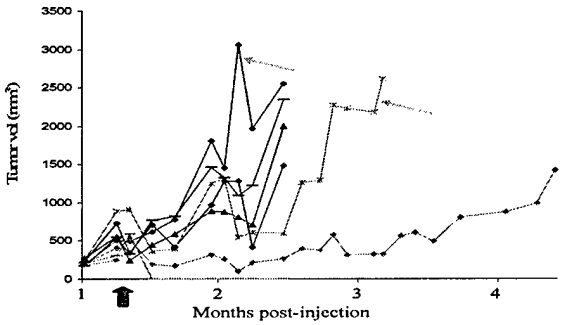
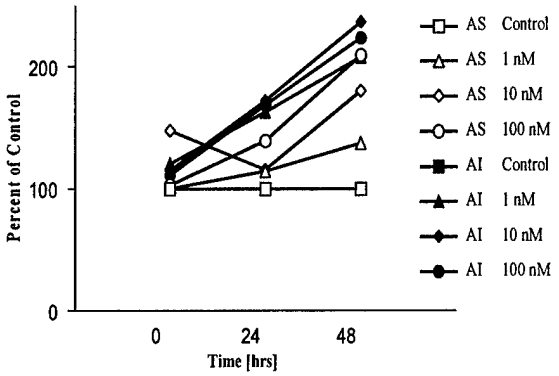



**Figure 5.** A graph of tumor volume over time plots the response of tumor growth rates in the presence and absence of androgen. Animals were castrated ~1.5 months (red arrow) after subQ implantation of pea size pieces of VCaP tumors. Pink and blue lines represent individual castrated and intact mice, respectively. The green arrow indicates animals further analyzed in Fig. 6. Tumor volumes were calculated from caliper measurements using the formula ($W^2$ x L x 0.526).

implanted with pea size tumor pieces. Tumor growth was measured using calipers twice per week and when a volume of 500-1000 $mm^3$ was reached half of the animals were castrated. Tumors continued to be followed in both groups and were harvested at 1 cM for reintroduction into castrated and intact mice as well as for reestablishment into culture and for RNA and protein preparations. For our first experiment, 8 animals were implanted under the flank with slivers of VCaP tumor, and 1.5 months later half of the animals were castrated. We predicted transient decreased rates of tumor growth or even reduced tumor volumes in the castrated animals following surgery while androgen sensitive cells were dying and androgen insensitive cells were establishing themselves. While this was observed shortly after castration (see Fig. 5), we also noted other timepoints of reduced tumor volume from the previous week's data and suspect variations in techniques for measuring tumor volumes were a contributing factor. As such, these

**Figure 6.** Growth rates of VCaP cells derived from a castrated mouse are relatively unchanged in the presence of androgen compared to cells harvested from an intact animal. Harvested tumors were dissociated by mild collagenase treatment to reestablish growth in culture. For DHT treatment, cells were grown in media free of phenol red with 10% charcoal-stripped FBS and supplemented with 0, 1, 10 or 100 nM DHT. WST assay was performed at the indicated times yielding a colorometric change directly proportional to cell number. Data was plotted as $A_{440}$ of treated cells relative to untreated controls.




experiments are being repeated both to apply more consistent techniques as well as to generate additional sublines for analysis. Preliminary analysis into the effects of cell growth in the presence of dihydrotestosterone (DHT) showed the growth rates of a VCaP subline derived from a tumor that reestablished after castration to be relatively unaffected by the presence of dihydrotestosterone (see Fig. 6). Conversely, a second subline harvested from an intact animal showed increased growth proportional to the amount of DHT added to the culture media. This experiment provides preliminary evidence that we have generated an AI VCaP subline and further characterization including AR expression and sequence studies are underway.

## KEY RESEARCH ACCOMPLISHMENTS

- Identification and preliminary characterization of PAR1 as a novel activator of the NF-κB signaling pathway and as a stimulator of IL-6 expression in prostate cancer cell lines.

- Isolation of a VCaP subline insensitive to the presence of androgen over a 2 log concentration range.

## REPORTABLE OUTCOMES

The manuscripts and abstracts listed below reflect the work accomplished with the support of this award. In addition, this award served as a springboard to a tenure track position for Dr. Cooper as an Assistant Professor of Biological Sciences at the University of Delaware-Newark. Personnel who have received pay from this research effort are:
Heather Muenchen, Ph.D. – Investigator
Carlton R. Cooper, Ph.D. – Investigator
Linda M. Kalikin, Ph.D. - Investigator
Kenneth J. Pienta, M.D. – Project Mentor

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## CONCLUSIONS

This continues to be a highly successful award that has resulted in key discoveries related to androgen receptor function and sensitivity to chemotherapy. Our bioluminescent murine metastatic prostate cancer model is ideal to investigate the *in vivo* effects of the PSA-IκBα super repressor on NF-κB and androgen receptor signaling as proposed in Specific Aim 3 of this project. Despite unexpected technical issues during this last year of funding (for which a swift resolution using new transfection technology is anticipated), connections between previously unlinked signaling pathways were discovered. In addition, prostate cancer sublines were generated that permit for the first time comparisons not only of an AI cell line directly against its AS parent cell line but also with a wildtype AR. In addition, VCaP and DuCaP provide the unique opportunity to analyze bone versus soft-tissue metastatic biology from the same patient. Thus we have generated a versatile and unique set of reagents with which to continue to investigate critical issues of prostate pathogenesis and AS to AI progression.

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## APPENDICES

Copies of publications and abstracts were provided in previous Annual Reports.