Title: Reactive Oxygen Species Produced by Prostate Cancer Cells Cause Castrate-Resistant Cell Growth by Inducing B-cell Lymphotoxin Release

Principal Investigator: Hirak S Basu, PhD

Contracting Organization: University of Wisconsin

Madison, WI 53715-1218

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Lack of a clear understanding of the mechanism of prostate cancer (PCa) progression should help drugs to prevent castrate-resistant PCa (CRPCa) progression in early-stage recurrent PCa patients. It has long been established that androgen signaling induces ROS production specifically in PCa cells by inducing a polyamine oxidation pathway. It has been shown that inflammatory response due to PCa causes release of specific cytokines that leads to B-cell infiltration in the PCa microenvironment. The B-cells, in-turn, switch on lymphotoxin (LT\(\alpha\) and/or LT\(\beta\)) production that has been implicated in ADPCa progression to CRPCa. Androgen-induced H2O2 released by PCa cells in the tumor microenvironment causes inflammatory response, B-cell infiltration and LT release leading to PCa progression to CRPCa. We proposed to co-culture PCa and human B-cells, where nutrient and metabolites can be exchanged freely. We proposed to take a novel and innovative microfluidic approach for the co-culture to closely mimic the in vivo microenvironment to reduce/eliminate the dilution effects observed in Boyden chambers. We studied if ROS produced by PCa cells can induce L Ts in B-cells that can help androgen-independent growth of ADPCa cells.
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INTRODUCTION

Progression of prostate cancer (PCa) from androgen-dependent (ADPCa) to androgen-independent castrate-resistant stage (CRPCa) is the Achilles’ heel in clinical management of PCa as well as developing effective therapy for early-stage progressing PCa patients. A clear understanding of the molecular mechanism of PCa progression should not only help develop novel therapy for patients with early-stage recurrence, but may also expand our basic understanding of the mechanism of PCa occurrence and recurrence leading to possible development of effective preventive agents.

It has long been known that PCa tissues are more oxidatively stressed than are normal prostate. It has been proposed that the cellular reactive oxygen species (ROS) play a critical role in PCa occurrence and progression [reviewed in 1,2]. Several publications have established that androgens induce ROS specifically in PCa cells. The increase in ROS levels is probably due to an induction of a polyamine oxidation pathway and specific small molecule inhibitors of this pathway prevent PCa growth both in culture as well as in transgenic animals [3]. We have shown that androgens cause upregulation of a transcription factor JunD in the prostate cancer cells [4,5]. Our published data demonstrate that JunD associates with activated androgen receptor (AR) in human PCa cells [6]. The resultant protein complex induces gene expression and consequent increase in the enzymatic activity of spermidine/spermine N1 acetyl transferase (SSAT) [6]. SSAT is the first enzyme that regulates a major spermidine/spermine (polyamine) oxidation pathway [reviewed in 7,8]. It converts the spermidine and spermine to their corresponding acetyl derivatives (shown in Figure 1). These products are oxidized by the constitutive FADH2-bound enzyme acetyl polyamine oxidase (APAO). APAO generates H2O2 during FADH2 ↔ FAD interconversion and releases bound FAD [9]. We have silenced SSAT expression by stably transfecting LNCaP cells with shRNA against SSAT (siSSAT) [3]. Unlike in the LNCaP cells, androgens fail to increase ROS in the siSSAT cells. It is known that ROS levels are higher in invading adenocarcinomas as compared to the normal epithelia [1,2]. It has been clearly demonstrated that ROS induce several functional proteins related to metastasis [10,11]. Thus, ROS may activate more than one mechanism to help androgen-dependent PCa (ADPC) cell survival and proliferation in the absence of androgen as well as its metastasis to distant organs leading to PCa progression to castrate-resistant PCa (CRPC). One of these inhibitors is now being developed for clinical testing.

Recently, it has been shown that inflammatory response due to PCa causes B-cell infiltration in the PCa microenvironment [12]. Activation of the transcription factor NF-kB leads to a paracrine induction of STAT3 and IL-6 in PCa cells that helps androgen-independent cell survival and proliferation in transgenic mouse model [13-15]. The mechanism of NF-kB activation in B-cells by prostatic inflammation is not well understood. We propose that androgen-induced polyamine oxidation produces high H2O2 in PCa cells that, when released in the tumor microenvironment, causes inflammation and B-cell infiltration as well as NF-kB activation in B-cells leading to PCa progression and metastasis. In order to test this hypothesis, we first tested genetically modified PCa cells with modified polyamine oxidation pathway and B-cells with modified lymphotoxins released due to NF-kB activation in cell culture system. As B-cells do not attach to any tissue culture plate, it is not possible to co-culture these two types of cells to create an in vitro tumor microenvironment, where free exchange of nutrient and metabolites can be exchanged between two types of cells.

To circumvent this problem, we used a microfluidic approach. It has recently been published that there is increased sensitivity in a microfluidic environment, when observing cell-cell signaling events [13]. Furthermore, tuning microchannel geometries results in the control of flow rates through a device during wash steps and causes shear stresses at the cell culture surface of the device. We used a novel microfluidic device, which leverages this tunability to facilitate B-cell co-cultures by limiting the shear stress experienced by the cells on the bottom of the culture chamber. This enabled washes and subsequent analysis of the non-adherent cell population. We observed that in this device, co-culturing with B-cells reverses the growth inhibitory effects of physiological androgen level on human PCa cells.

BODY

Methods

Cells and growth media: As proposed, LNCaP human PCa cells and LNCaP cells stably transfected with shRNA against SSAT (siSSAT) were used for these studies. While LNCaP cells produce reactive oxygen species (ROS) in the presence of androgen, siSSAT cell line does not and is used as a negative control. All cells were grown in RPMI-1640 medium containing either 10% FBS or 1% FBS and 9% Charcoal-Stripped Serum (CSS) to maximize androgen receptor expression.

Microfluidic Device: We used a microfluidic co-culture device (shown schematically in Figure 2) to culture human PCa cells and human B-cells that are connected through exchange of fluid, but no cell-cell contact. In some experiments the PCa cells were cultured in the outside wells and B-cells in the center well, in other experiments the seeding chambers were reversed. In yet other experiments, the conditioned
media from the B-cell cultures were used. For the control experiments the PCa cells were grown without the B-cells or conditioned media from B-cells.

**Results**

**Aim #1.** To test if the $\text{H}_2\text{O}_2$ (oxidative stress) produced by androgen-treated LNCaP cells induces LT release by human B-cells.

To test this hypothesis, we first standardized the microfluidic device for cell growth. The standard device material used for fabricating the microfluidic device was found to be not useful for these studies due to non-specific absorption of androgen and synthetic androgen R1881 on the surface. After testing several materials, we standardized the use of the material suitable for these studies to fabricate these microfluidic channels, where LNCaP cells exhibit the androgen induced changes in cell growth similar to what reported in the literature [3-5]. A representative picture of LNCaP cells grown under these conditions are shown in Figure 3a. The DNA fluorescence intensity data after staining the cells with DNA-binding fluorescence dye Syto 60 are shown in Figure 3b. The effect of R1881 on cell growth in this device is similar to that in a 96 well co-star plate based assay, albeit at a higher R1881 concentration. This difference in R1881 requirement could be due to one or more of the following reasons – a) some absorption of R1881 on the plate material, b) the constraints on the cells in a microfluidic environment, or c) difference in seeding densities in microchannels from that in a 96 well plate based assay. Because of the additional time to standardize this assay, we did not perform the lymphotoxins production assay in human B-cells as proposed. Instead, we used Ramos human B-lymphoma cell line that constitutively produces both lymphotoxins $\alpha$ and $\beta$ [16].

**Aim #2.** To test if the release of the LTs by B-cells promotes androgen-independent growth of ADPCa cells.

For these studies, we standardized growth conditions for co-culturing Ramos cells with LNCaP or siSSAT cells. A low magnification picture of the co-culture in the microfluidic chamber (shown schematically in Figure 1) is shown in Figure 4. We have also standardized methods for growing Ramos cells in the outside chambers and PCa cells in the center well. We did not find any difference in cellular characteristics, if the growth chambers are interchanged (data not shown). We used one or the other set-up depending on the study requirements.

We then studied the growth of PCa cells, when co-cultured with Ramos cells or conditioned media from Ramos cells grown for at least 48 h. A representative picture of the cells grown with or without Ramos cells in the microfluidic chamber is shown in Figure 5a. The growth data calculated from the Syto60 staining intensities of the nuclei are shown in Figure 5b. It is evident that the growth inhibitory effect of androgen observed here in the microfluidic chamber and has been reported over the last decade from our and other laboratories in standard 96-well plate based assay is reversed in microfluidic device, when co-cultured with Ramos B-cells or conditioned media from the B-cell culture.
Since we used Ramos human B-lymphoma cells that are constitutively producing LTβ, instead of using CMV promoter to induce high expression of LTs in B-cells as proposed, we transfected an expression vector containing shRNA against LTβ to silence the expression of LTβ in these cells. The qRT-PCR data for silencing LTβ expression in Ramos cells are shown in Table 1. The shRNA reduced LTβ expression by nearly 2 cycles, which is about 25% of that expressed in the control cells.

We compared the effects on the growth of LNCaP cells using the conditioned media from LTβ silenced Ramos cells grown for at least 48 h in the adjacent chamber. No difference of LNCaP cell growth was observed in cells grown with the conditioned media either from the wild-type Ramos cell or from the LTβ-silenced Ramos cells (data not shown). These experiments are currently being repeated for confirmation.

Lastly, we asked if changes in cellular oxidative stress often observed in PCa cells are responsible for the growth effect of the B-cells as shown in Figure 5b. As proposed, the oxidative stress is measured by a dichlorofluoresceine diacetate (DCF-DA) dye oxidation assay previously standardized in our laboratory in a 96-well plate based assay. This assay has now been standardized in the microfluidic system. The green fluorescence of DCF dye oxidation in PCa cells seeded in the center well is shown in Figure 6a (top panel) along with the red fluorescence of nuclear staining by DNA-binding dye TO-PRO-3 (bottom panel). The PCa cells were treated with R1881 for 48 h in the presence and absence of the conditioned media from Ramos cells in the adjacent well. The fluorescence intensity ratios of DCF fluorescence to that of TO-PRO-3 fluorescence are shown in Figure 6b. Unlike 96-well plate based assay, there was no observable effect of androgen on the cellular ROS levels, neither was it affected by the presence of Ramos cell conditioned media. In view of the negative results the proposed studies with normal B-cells from human tonsils and use of siSSAT cell line were not performed.

### KEY RESEARCH ACCOMPLISHMENTS

**A)** It has long been reported from 96-well based studies carried out in several laboratories that androgen, at or near physiological concentration, causes growth inhibition of androgen-dependent prostate cancer cells. These results cannot explain the **in vivo** and clinical observation of the growth of androgen-dependent PCa in androgen sufficient animals and humans. The data shown in Figure 5 clearly demonstrate that androgen-dependent PCa cell proliferation can continue normally in presence of infiltrating B-cells at the inflammatory zone of the PCa. Thus, the microfluidic device for PCa cell growth in the presence of B-cells more accurately mimic the **in vivo** conditions and can be used for future **in vitro** studies that can be quickly translated to a clinical setting. We have used this information to set-up experiments to identify prognostic biomarkers in the PCa cells that have now been extended to human biopsies and prostatectomy tissues.

**B)** The data presented in Figure 5 also show that the conditioned media from the B-cell have the exact same effect as culturing B-cells in the adjacent chambers. Thus, the B-cell factor(s) that help(s) PCa cell proliferation in the presence of androgen is a paracrine, serum soluble factor(s). These data also support the published **in vivo** activated lymphotoxins released from the B-cells act as paracrine factors to activate the NF-κB mediated cell proliferation of castrate-resistant PCa. Our data using LTβ silenced B-cells did not affect PCa cell growth. This difference from the **in vivo** observation may be due to – (a) LTα instead of LTβ is the paracrine factor that drives the CRPCa growth, (b) silencing down to 25% may not be enough to abrogate the growth effect as LTs are very good activators of NF-κB, (c) some other paracrine factor(s) other than LT is (are) responsible for the proliferative effect. The serum soluble factor may be isolated by fractionation of the conditioned media that are now being contemplated.

### Table 1

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C) We have proposed that the reactive oxygen species (ROS) produced by the PCa cells affect the release of the proliferation factor(s) from the B-cells. Our data shown in Figure 6 demonstrated no difference in ROS production in PCa cells with or without B-cell media. Thus, we believe the role of ROS, if any, is exhibited during PCa formation that causes the inflammation and B-cell migration. Once the B-cells are within the cancerous region the LTs or other paracrine factors released by the B-cells are sufficient to induce cell proliferation. Interestingly, the ROS levels seen in PCa cells in the microfluidic device are not affected by the growth inhibitory concentrations of androgen. This is different from what is observed in a 96-well plate based assay [3-5]. This may open up an avenue of testing androgen-induced PCa cell growth without affecting cellular ROS levels. The full implication of the use of such an in vitro model and its relevance to clinical observation of PCa progression is now under consideration.

REPORTABLE OUTCOMES
Standardization of the cell culture material for microchannel devices that are suitable for performing studies with androgen effects on prostate cancer is a major achievement in this project. This is not only applicable for studying androgen effects in prostate cancer in microchannels, it may also be used for testing estrogen and other lipid soluble hormones and drugs in prostate, breast, ovarian and other cancer cell lines. A full RO1 proposal related to clinical validation of biomarkers based on data generated using such microchannels has been submitted to NIH for funding.

A manuscript is now being written up using data generated under this proposal and other studies carried out in these microchannel devices for publication. A copy of the publication will be made available to DoD, if requested.

CONCLUSION
In conclusion, the studies carried out under this project will help progress the research in the field of hormonal effects on human cancers in an in vitro mimetic of the in vivo scenario. This will have far reaching consequence in the field of drug development, biochemical and mechanistic studies on tumor progression as well as identifying human malignant cells with high risk of progression in human tumor biopsies. The preliminary data for the field of tumor prognosis, although not generated in this project, have been presented by the PI upon invitation from the AACR-NCI-EORTC meeting “Molecular Targets for Cancer Therapeutics” in November 2012. We believe, the in vitro microfluidic mimetic device used in this project will be a major contributor to the clinical prognosis of prostate cancer.

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PERSONNEL

Full-time personnel who received pay from this research effort include:

Basu, Hirak S.

Beebe, David J.

Church, Dawn R.

Mehraein, Farideh

Myers Y Gutierrez, Adan L.

Su, Xiaojing

Weichmann, Ashley M.

Wilding, George
APPENDIX

Abstract of Presentation at EORTC-AACR-NCI meeting November, 2012

Activation of a specific metabolic pathway may distinguish aggressive from indolent prostate cancer

Wei Huang1, Pamela A. Young2, Kevin W. Eliceiri2, George Wilding3 and Hirak S Basu3

1Department of Pathology and Laboratory Medicine, 2Laboratory for Optical and Computational Instrumentation, 3Department of Medicine and Public Health
University of Wisconsin Carbone Comprehensive Cancer Center, Madison, WI

Background: Persistent increase in serum prostate specific antigen (PSA) levels is an accepted indicator of prostate cancer (PCa). Other prostatic diseases may also elevate serum PSA levels. Thus, it has less than 30% specificity for PCa. Also, a widespread PSA screening is leading to the diagnosis of many low grade and small PCas. Some of these PCas may progress and become lethal, but many of them may remain indolent for the rest of the patients’ lives. There is no approved biomarker that can distinguish the aggressive PCas from the indolent ones.

There is strong evidence that reactive oxygen species (ROS) play a key role in the recurrence of androgen-dependent PCa (ADPC) and its progression to often lethal castrate-resistant PCa (CRPC) [1]. It has been shown that androgen induces an increase in ROS levels in cultured ADPC cells via a yet unknown mechanism [2]. We have demonstrated that in certain human PCa cells, androgen causes a marked overexpression and consequent increase in the enzymatic activity of spermidine/spermine acetyl transferase (SSAT) [3]. SSAT converts the polyamines spermidine and spermine to their corresponding acetyl derivatives. An oxidation of acetyl-polyamines by FAD-bound acetyl polyamine oxidase (APAO) is a major contributor to the androgen-induced ROS production in polyamine-rich PCa cells. Cellular ROS can set-up an autocrine feed-forward loop of SSAT-ROS-NF-κB-SSAT. This loop keeps on producing ROS activating AR in the absence of androgen and thus helps progression of ADPC to CRPC.

Methods: We used multiphoton fluorescence microscopy to determine FAD fluorescence intensity and lifetime in human prostate biopsies containing both PCas and normal prostate tissues. We also used RNA-in situ hybridization (RNA-ISH) study of SSAT gene expression in two human prostate tissue microarrays containing 384 and 462 samples.

Results: There is a marked increase in FAD fluorescence intensity and decrease in the ratio of protein-bound FAD to free FAD in PCas as compared to normal epithelia. This suggests increased activity of an FAD-bound enzyme that recycles FADH2 to increase the cellular free FAD levels. The data showed a marked increase in SSAT expression in PCas as compared to that in the normal prostatic epithelia (P<0.001). More interestingly, an even higher SSAT expression in metastatic PCas relative to localized PCas (P=0.014) was also observed.

Conclusion: Multiphoton microscopy to determine FAD fluorescence and SSAT gene expression may be employed clinically for PCa prognosis.

References: