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Development of the VCaP Androgen Independent Model of Prostate Cancer

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Abstract

Prostate epithelial cell growth is dependent on the presence of androgens and the transition of prostate cancer to an androgen independent phenotype results in a highly aggressive, currently incurable cancer. We have developed a new preclinical model of androgen independent prostate cancer derived from the VCaP prostate cancer epithelial cell line. VCaP cells were subcutaneously implanted and serially passaged in castrated male SCID mice. Androgen independence was confirmed by WST-1 (a tetrazolium salt) cell proliferation assay in the absence or presence of dihydrotestosterone (DHT) (1 – 100 nM). VCaP androgen sensitive (VAS) cells responded dose dependently to DHT whereas VCaP androgen independent (VAI) cells did not alter their proliferation in response to DHT. Gene expression of androgen receptor, Bcl-2, DD3, prostate acid phosphatase, STEAP, and survivin was determined by PCR amplification. Bcl-2 expression was upregulated in the VAI lines compared to the VAS suggesting a possible mechanism of androgen independence. Furthermore, tumor – associated – angiogenesis was assessed by immunofluorescence confocal microscopy of CD31. VAI tumors demonstrated enhanced angiogenesis compared to VAS tumors. These results demonstrate the development of a novel model of prostate cancer androgen independence and provide a new system to study angiogenesis and the transition to androgen independence.

Keywords

prostate; cancer; androgen independent; VCaP

1. Introduction

The primary treatment for patients with prostate cancer who have failed primary therapy is androgen deprivation therapy (ADT). The majority of advanced prostate cancer tumors that recur are initially responsive to androgen blockade. However, nearly 100% of patients undergoing ADT fail hormone therapy and progress to a more aggressive androgen independent (AI) cancer phenotype (Figure 1A). Uncovering the molecular events that underlie the conversion of prostate cancer to the AI phenotype is one key to developing further treatment strategies in androgen independent prostate cancer (AIPCa).

Multiple mechanisms have been identified that contribute to the androgen sensitive (AS) transition to the AI phenotype [1–5]. In the outlaw pathway, receptor tyrosine kinases are

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activated, and the androgen receptor (AR) is phosphorylated by either the AKT (protein kinase B) or the mitogen-activated protein kinase (MAPK) pathway, producing a ligand-independent AR. Grossman et al (2001) demonstrated activation of the AR receptor by protein kinase A (PKA), as well as, Her2/Neu, a receptor tyrosine kinase [6]. Similarly, Navarro et al (2002) reported that insulin-like growth factor-1 (IGF-1), epidermal growth factor (EGF) and keratinocyte growth factor (KGF) induced AR receptor activation [6,7]. Additionally, evidence suggests that activation of two predominant signaling pathways (Akt and MAPK pathways) may contribute to androgen independent growth. Kousteni *et al.* (2001) demonstrated that estrogen receptor and the androgen receptor can associate in the same complex and can protect osteoblasts and osteocytes from apoptosis through the Src/Shc/ERK signaling pathway [8]. In the promiscuous pathway, the specificity of the AR is broadened so that it can be activated by non-androgenic molecules normally present in the circulation. In the hypersensitive pathway, more AR is produced (usually by gene amplification), or AR has enhanced sensitivity to compensate for low levels of androgen, or more testosterone is converted to the more potent androgen, dihydrotestosterone (DHT), by 5 α -reductase. In the bypass pathway, parallel survival pathways, such as that involving the anti-apoptotic protein Bcl-2 (B-cell lymphoma 2) obviate the need for AR or its ligand. In the stem cell repopulation pathway, androgen-independent cancer stem cells are resistant to therapy and eventually grow out and eventually become the primary population within the tumor.

The lack of appropriate, clinically relevant, cell-based model systems has limited prostate cancer research and the development of new therapeutic modalities. We have previously isolated and characterized a new adherent prostate cancer cell line derived from a vertebral metastatic lesion (VCaP) [9]. The cells were characterized for tumorigenicity, doubling time, karyotype, and for expression of cytokeratin-18, wild-type androgen receptor (AR), androgen sensitivity (AS), prostatic acid phosphatase (PAP), prostate specific membrane antigen (PMSA), prostate specific antigen (PSA), Rb, and p53 [9]. We have utilized the VCaP model system to generate an androgen independent subline (VAI) to further understand the transition to the androgen independent phenotype. The novelty of this model system is the expression of a wildtype AR in the VCaP androgen sensitive (VAS) line and its ability to be passaged both within animals as well as in cell culture.

2. Materials and methods

2.1. Generation of VCaP AI by castration of xenograft-implanted SCID mice

VCaP tumors were placed subcutaneously into the flank of 10 male SCID mice. MRI images and quantification of tumor volume using calipers was performed weekly to monitor and track tumor growth (tumor volume = $L \times W \times W \times 0.56$). Once the tumors reached a grossly measurable size of 300–400 mm³, 5 mice were left intact and 5 mice were castrated. The tumors were allowed to involute and regrow in the castrated animals. Tumors were allowed to grow 8 weeks before passage into culture or continued in castrated mice. Single cell suspensions were generated by dissecting away the tumor, placing it into a 100 mm Petri dish, and rinsing in Medium 199/2x antibiotic/mycotic. The media was aspirated off and the tumors were resuspended in DMEM. After macerating the tumor, 1 mL of 10x collagenase was added for further digestion of the tumors. The samples were transferred to a Petri dish and incubated for 30 minutes at 37C. Fetal bovine serum (20% v/v) was added to inhibit further collagenase digestion, and the remaining sample was collected by centrifugation. The supernatant was discarded and the pellet was resuspended in 15 mL of culture media and transferred to a 100 mm culture dish. Alternatively, the tumors were placed in DMEM + 10 % fetal bovine serum + 1 % antibiotic/antimycotic and washed 3 times. Tumors were diced into small pieces and placed in fresh media at 37C in 5% CO₂ for future passage.

2.2. Cell culture of VAS and VAI sublines

VAS cells were maintained in DMEM (Invitrogen Corp.) + 10% FBS + 1% antibiotic/antimycotic. VAI cells were maintained in phenol red free DMEM (Invitrogen Corp.) + 10% charcoal stripped FBS (HyClone) + 1% antibiotic/antimycotic.

2.3. Proliferation rates by WST-1 assay

Cells were seeded at a density of 5×10^5 cells/well in a six well plate in regular media. Twenty four hours after seeding the media was changed with half of the wells receiving regular media and half receiving androgen-reduced media (phenol red-free DMEM media supplemented with 10% FBS and 1% antibiotic/antimycotic). Cell growth was determined at 0, 24, and 48 hours after seeding using the WST-1 assay (Pierce Biotech). Co-experiments in the presence of dihydrotestosterone (DHT) were performed in AS cells and AI cells separately. DHT induced the growth rate of androgen sensitive cells with minimal effects on androgen insensitive cells.

2.4. PCR Analysis

cDNA was synthesized from 5 ug total RNA using the First Strand Superscript cDNA synthesis kit (Invitrogen Corp) following manufacturer's instructions. Standard PCR techniques were used to detect gene expression. RNA integrity was determined using Agilent technology prior to cDNA synthesis reactions (Agilent Inc.). PCR reactions contained 3 ul of 10x reaction buffer containing MgCl₂, 3 ul of 2 mM dNTP mix, 2 ul of the sense and antisense primers at 50 ng/ul each, 2 ul cDNA, and 0.2 ul Taq in a total volume of 30 ul. Gene specific primers and annealing temperatures are outlined in Table 1. Amplification products were separated on a 1% agarose gel in 1x TBE and visualized with ethidium bromide staining.

2.5. Immunofluorescence

Subcutaneous tumors were removed and snap frozen in liquid nitrogen prior to analysis. Frozen tumors were submerged in OCT media and stored at -80°C prior to sectioning. Five μm tissue sections were cut and placed on glass slides for immunofluorescent analysis. Slides were washed once in 100% ethanol for 5 min and rehydrated twice in tris-buffered saline (TBS) for 5 min each. Slides were blocked in 3% BSA in TBS + 0.05% Tween 20 (TBS-T) for 60 min and rinsed once with TBS-T for 5 min. Primary antibody (FITC conjugated anti-CD31) was diluted 1:100 in TBS-T + 1% BSA and applied to the sections for 60 min in a humidified chamber. Slides were washed three times with TBS-T for 15 min each. Nuclei were stained with DAPI containing ProLong Gold Antifade Reagent (Molecular Probes, Inc) and kept at 4°C for further analysis. Images were captured as 24-bit TIFF files using an Olympus FV-500 Confocal microscope and a 20x objective.

3. Results

3.1 Generation of VCaP androgen independent cells

We have created a new preclinical model for studying the transition from AS to AI using the previously described VCaP model of prostate cancer [9]. A parental line of VCaP prostate cancer cells were implanted subcutaneously into 10 5–6 week old male SCID mice. Two weeks post-injections 5 mice were castrated and the tumors were allowed to involute and regrow. Tumors implanted in intact mice demonstrated an increase in volume over a 60 day period (Day 1: $37.3 \pm 6.69 \text{ mm}^3$, Day 52: $2092.7 \pm 469.32 \text{ mm}^3$; mean $\pm$ SD). Five mice were castrated 15 days post-implantation (Figure 1B). Tumors in the castrated mice began to involute immediately post-castration and remained dormant for an average of 14 days. Tumors regrew over a 74 day period (Day 1: $26.99 \pm 4.3 \text{ mm}^3$, Day 74: $1270.41 \pm 333.87 \text{ mm}^3$; mean $\pm$ SD) before the mice were sacrificed and tumors were passaged subcutaneously in additional castrated SCID mice and placed in culture by collagenase digestion. One mouse was lost during

surgery and two mice were lost during the 74 day tumor expansion period. Cells from VAS11 and VAI32 (Figure 1B) were successfully transferred to cell culture.

3.2 WST-1 proliferation assay comparing VAS vs. VAI cell growth

Androgen independence of the VAI32 cells was assessed via the WST-1 cell proliferation assay in the presence or absence of dihydrotestosterone (DHT) (Figure 2). VAS11 cells did not proliferate in androgen free media but increased their rate of proliferation dose dependently to increasing concentration of DHT after 48 hours (Control: 0.178 ± 0.02 , DHT 1 nM: 0.228 ± 0.05 , DHT 10 nM: 0.284 ± 0.06 , DHT 100 nM 0.322 ± 0.02 ; mean $\pm$ SD). VAI32 cells demonstrated androgen independent and insensitive growth in the absence of androgen and in the presence of increasing concentration of DHT after 48 hours (Control: 0.260 ± 0.03 , DHT 1 nM: 0.259 ± 0.02 , DHT 10 nM: 0.275 ± 0.04 , DHT 100 nM 0.269 ± 0.03 ; mean $\pm$ SD). Additionally, VAS11 and VAI32 lines have been serially passaged subcutaneously in mice for 6 generations. VAI32 cells have also been maintained viable in culture and passaged successfully for 12 passages in an androgen depleted environment (data not shown).

3.3 Differential mRNA expression of prostate markers by PCR in VAS vs. VAI

We examined the differential mRNA expression pattern of several prostate markers to demonstrate that the AI line maintained a prostate epithelial cell phenotype. Table 1 lists the primers and conditions used for PCR amplification of the AR, Bcl-2, DD3, PAP, STEAP, Survivin, and β -actin. Increasing cycles were used to detect mRNA expression. No appreciable difference was observed in the expression of AR, PAP, STEAP, and survivin between VAS11 and VAI32 cell lines. There was a noticeable qualitative increase in mRNA expression of Bcl-2 and DD3 in the VAI32 cell line compared to the VAS11 cell line.

3.4 Immunofluorescence of CD31 in VAS vs. VAI frozen tumor sections

At the time of collagenase digestion, VAS11 and VAS32 tumor specimens were snap frozen in liquid nitrogen for immunofluorescent analysis. We examined the ability of the AI tumor to induce angiogenesis by probing for CD31, a marker for angiogenesis also known as PECAM-1 (platelet-endothelial cell adhesion molecule). VAI demonstrated increased staining of CD31 positive neovascularization compared to the VAS tumors (Figure 4).

4. Discussion

Several models of androgen independent prostate cancer have previously been reported [10–15]. Igawa et al analyzed early passage (< 30) vs. late passage (< 80) LNCaP cells and demonstrated the development of androgen independence in the late passage cell lines [13]. Similarly, Zhou et al orthotopically injected LNCaP cells into castrated mice and passaged these tumors for 5 generations, each generation resulted in a more aggressive androgen independent tumor [11]. Wu et al developed the C4-2B model of androgen independence from a bone derived subline of LNCaP cells [16–18]. Trepper et al developed a CWR22 hormone-independent variant, CWR22-R, Klein et al generated the LAPC model of AIPCa in SCID mice, Navone et al established the PCa2a and PCa2b model of AIPCa, and Corey et al developed the LuCaP xenograft model of AIPCa [10,12,14,15]. All of these models have provided valuable insight into the development of androgen independent disease, however additional models can provide insights into prostate tumorigenesis. We have developed a new preclinical *in vivo* and *in vitro* model to identify and characterize mechanisms that contribute to the transition to androgen independence.

VASand VAI cells can be passaged in serial xenografts as well as in culture. The parental VCaP cell line was shown to express the wild type androgen receptor (AR) which created a unique model to investigate AR in the transition to androgen independence [9]. Currently, we have

demonstrated that VAS and VAI cells express AR and no significant difference in expression was observed (using standard PCR techniques) in AR mRNA between VAS and VAI cells. Additionally, VAI cells expressed PAP, STEAP, and DD3 which are known prostate markers and have been associated with tumor progression [19–21]. VAI cells demonstrated enhanced expression of Bcl-2 compared to VAS cells. Increased Bcl-2 expression has been implicated as a mechanism of androgen independence and drug resistance in prostate cancer [22,23]. Comparing the sensitivity of chemotherapeutic agents in the VAS vs. VAI cells may provide a new platform for studying drug resistance in prostate cancer cells.

Immunofluorescent staining revealed increased neovascularization in VAI tumors compared to VAS tumors. Increased angiogenesis in androgen independent tumors has previously been described [24]. Gustavsson et al reported an increase in microvascular density and morphology in tumors derived from the LNCaP-19 model, an androgen independent subline [25]. The development of androgen independence, as well as enhanced angiogenesis is associated with advanced, highly aggressive prostate cancer. Androgens have been implicated in the regulation of endothelial cells and tumor vascularization and androgen ablation has been shown to induce endothelial cell apoptosis in prostate endothelial cells [26]. The ability of endothelial cells to escape androgen ablation-induced apoptosis may be an important mechanism resulting in the transition of to an androgen independent phenotype and may identify an alternative avenue for treating androgen independence.

In summary, the VCaP androgen independent cell line presented here provides a unique opportunity to investigate the transition to an AI phenotype, as well as the role of AI in tumor aggressiveness. This model will lead to a better understanding of the biology of AI and may be useful in identifying potentially novel mechanisms of AI development and therapeutic targets for the treatment of prostate cancer.

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Figure 1.

Generation of a VCaP androgen independent cell line. A) A theoretical depiction of the transition to androgen independence after androgen deprivation therapy. [AS – androgen sensitive prostate cancer; AI – androgen independent prostate cancer] B) Tumor volumes of representative xenograft VCaP implants that were successfully transferred to cell culture. Mice were subcutaneously injected with VCaP cells on Day 0. Five mice were castrated (indicated by the arrow) on Day 16 and 5 mice were left intact. [AS11 – an intact mouse with a xenograft transferred to culture; AI32 – a castrated mouse with a xenograft transferred to culture]

Figure 2.

WST-1 proliferation assay in the absence or presence of dihydrotestosterone (DHT). VAS (AS11) and VAI (AI32) cells were plated in androgen-free media and supplemented with increasing concentrations of DHT (1 to 100 nM) for 24 to 48 hours. Open symbols represent the VAS cells (□ – AS untreated, Δ – AS DHT 1 nM, ◊ – AS DHT 10 nM, ○ – AS DHT 100 nM; ▪ – AI untreated, ▲ – AI DHT 1 nM, ◆ – AI DHT 10 nM, • – AI DHT 100 nM).

Figure 3. mRNA expression analysis of VAS and VAI cells by PCR. Five μ g RNA of VAS and VAI cells from culture were used to synthesize cDNA. PCR amplification was performed at 25, 30, and 35 ($\blacktriangle$) cycles and samples were visualized on a 1% agarose gel using ethidium bromide. [AR – androgen receptor; DD3 – prostate cancer antigen 3; PAP - prostatic acid phosphatase; STEAP – six transmembrane epithelial antigen of the prostate; Bcl-2 – B-cell lymphoma 2; surviving - apoptosis inhibitor 4]

Figure 4. Immunofluorescence of CD31 positive neovascularization between VAS and VAI tumors. Frozen tumor specimens were cut into 5 μ m sections and labeled with an anti-CD31 antibody and DAPI to visualize the tumor vasculature. Images were captured using confocal microscopy and a 20x objective. A) VAS nuclear stain – DAPI; B) VAS anti-CD31; C) VAS overlay; D) VAS magnification of overlay; E) VAI nuclear stain – DAPI; F) VAI anti-CD31; G) VAI overlay; H) VAI magnification of overlay.

Table 1

PCR primers and annealing temperatures

Gene	Sense	Antisense	Tm	bp
Actin	CCTCGCCTTTGCCGATCC	GGATCTTCATGAGGTAGTCAGTC	52	626
AR	CATCCTGCTCAAGACGCTTC	CACAGAGATGATCTCTGACA	52	155
Bcl-2	TCATGTGTGTGGAGAGCGTCAA	CTACTGCTTTAGTGAACCTTTTGC	55	306
DD3	AGATTGTGGTGTGCAGCC	TCCTGCCCATCCTTTAAGG	55	360
PAP	ATGGAGCAGCATTATGAACTTGGAG	GCAAGAAGCATAGGGAGGAAGGA	55	716
STEAP	CTGATGAATTTGACTGCCCT	TACTGGCTCTGTTGGCTGTG	52	550
Survivin	GCATGGGTGCCCCGACGTTG	GCTCCGGCCAGAGGCCTCAA	55	431