The Androgen Receptor Induces Integrin α6β1 to Promote Prostate Tumor Cell Survival via NF-κB and Bcl-xL Independently of PI3K Signaling

Laura E. Lamb1, 2, Jelani C. Zarif1, 2 and Cindy K. Miranti1

1Laboratory of Integrin Signaling and Tumorigenesis, Van Andel Research Institute, Grand Rapids, MI
2Cell and Molecular Biology Program, Michigan State University, East Lansing, MI

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*Corresponding Author:
Cindy Miranti, Ph.D.
Van Andel Research Institute
333 Bostwick Ave NE
Grand Rapids, MI 49503
Phone: 616-234-5358
Fax: 616-234-5359
cindy.miranti@vai.org
ABSTRACT

Recent studies indicate that androgen receptor (AR) signaling is critical for prostate cancer cell survival; even in castration-resistant disease where AR continues to function independently of exogenous androgens. Integrin-mediated adhesion to the extracellular matrix is also important for prostate cell survival. AR-positive prostate cancer cells express primarily integrin α6β1 and adhere to a laminin-rich matrix. In this study, we show that active nuclear-localized AR protects prostate cancer cells from death induced by PI3K inhibition when cells adhere to laminin. Resistance to PI3K inhibition is mediated directly by an AR-dependent increase in integrin α6β1 mRNA transcription and protein expression. Subsequent signaling by integrin α6β1 in AR-expressing cells increased NF-κB activation and Bcl-xL expression. Blocking AR, integrin α6, NF-κB, or Bcl-xL concurrent with inhibition of PI3K was sufficient and necessary to trigger death of laminin-adherent AR-expressing cells. Taken together, these results define a novel integrin-dependent survival pathway in prostate cancer cells that is regulated by AR, independent of and parallel to the PI3K pathway. Our findings suggest that combined targeting of both the AR/α6β1 and PI3K pathways may effectively trigger prostate cancer cell death, enhancing the potential therapeutic value of PI3K inhibitors being evaluated in this setting.
INTRODUCTION

Androgen, acting through the androgen receptor (AR), is required for prostate cancer growth and survival. Therefore, chemical castration is initially an effective treatment for advanced prostate cancer. However, patients ultimately relapse with castration-resistant tumors for which there are no effective treatments. Nonetheless castration-resistant tumor cells are still dependent on AR, as inhibition of AR expression leads to cell death (1-3). How AR regulates survival of castration-resistant tumor cells is poorly understood.

Integrins are heterodimeric cell surface receptors that mediate cell survival through adhesion to extracellular matrix (4, 5). Integrin signaling through various pathways regulates pro-survival and pro-death molecules and matrix detachment induces cell death (6). Integrin expression and signaling is aberrant in many cancers, including prostate cancer. In the normal human prostate, basal epithelial cells express two integrins, α6β4 and α3β1, which promote basal cell survival through adhesion to laminin 5 in the basement membrane (7, 8). Basal epithelial cells do not express AR, but differentiate into AR-expressing secretory cells which down-regulate integrins and no longer adhere to the basement membrane (9). Thus, integrin and AR expression are mutually exclusive in normal prostate epithelium. However, in prostate cancer the AR-expressing tumor cells exclusively express integrin α6β1 and adhere to a remodeled matrix containing the α6β1-specific substrate, laminin 10 (10, 11). The predilection for α6β1 expression is preserved in lymph node metastases (12). Constitutive AR expression in immortalized prostate epithelial cells increases integrin α6 (13), suggesting that AR could be responsible for maintaining α6 expression in the cancer cells. In addition, the α6 promoter contains a steroid-response element capable of stimulating α6 expression in response to progesterone (14). Thus, AR-mediated control of integrin α6 and the engagement of α6β1 in AR-expressing cells could provide a novel mechanism for prostate cancer cell survival.

Phosphoinositide 3-kinase (PI3K) signaling is required for survival of most prostate cancers. PTEN, a phosphoinositide phosphatase and negative regulator of PI3K signaling, is lost in ~30% of clinical prostate cancers and in ~60% of metastatic cancers, resulting in constitutive activation of PI3K (15, 16). Akt is a major downstream effector of PI3K signaling and regulates survival through inhibition of pro-death proteins, such as Bad, Bax, FOXO, DAP3, and caspase 9, and increased expression of the pro-survival protein survivin and stimulation of NF-κB and mTOR signaling (6, 17). Nonetheless, PI3K signaling is not the only survival pathway. The androgen-sensitive prostate cancer cell line LNCaP dies upon PI3K/Akt inhibition; however, addition of androgen can rescue this death (18, 19). In addition, long term androgen ablation results in resistance to PI3K/Akt inhibition (20) and prostate regeneration
studies demonstrate that AR and Akt can synergize to promote tumor formation even after androgen ablation (21). This suggests that AR, and in some contexts independent of exogenous androgen, promotes survival independent of PI3K. In this study, we tested the hypothesis that AR-dependent regulation of integrin α6β1 expression in prostate cancer cells promotes survival independent of PI3K.

MATERIALS AND METHODS

Cell Culture. PC3, DU145, LNCaP and VCaP cells authenticated by DNA profiling were obtained from ATCC. PC3 cells were grown in F-12K containing 10% charcoal-stripped and dextran-treated FBS (CSS). DU145-AR cells were grown in MEM Earles containing 10% CSS, non-essential amino acids, and sodium pyruvate. LNCaP cells were grown in RPMI-1640 supplemented with 10% FBS, 0.225% glucose, 10mM HEPES, and sodium pyruvate. VCaP cells were cultured in DMEM with sodium pyruvate and 10% FBS. An original stock of C4-2 cells was obtained from Dr. Leland Chung (22) and grown in RPMI-1640 and 10% FBS. LNCaP, C4-2, and VCaP cells were grown in phenol red-free media and 10% CSS 48 hours prior to experimental use. For all experiments, cells were plated on 10µg/mL Laminin 1 (Invitrogen) (8, 23).

DNA Constructs. pBabe-puro-hAR and pGL3-vector plasmids were provided by Dr. Beatrice Knudsen. pCSCG-AR-ΔNLS and pCSCG-AR-N705S (ΔLBD) plasmids were obtained from Dr. Owen Witte (21, 24). pLKO.3pg was provided by Dr. Jeff MacKeigan. pBabe-puro-Bcl-xL was a gift from Dr. Douglas Green. pGL4.32-luc2P/NF-κB-RE and phRG-TK were purchased from Promega. All AR plasmids were sequenced verified. PC3-Puro, DU145-Puro, PC3-AR, DU145-AR, and PC3-Bclxl cells were generated by infecting cells with pBabe-puro, pBabe-puro-hAR, or pBabe-puro-Bclxl retroviruses. Clones were selected and maintained in 2µg/mL puromycin. PC3-pLKO, PC3-ΔNLS, and PC3-ΔLBD cells were made by infecting cells with pLKO.3pg, pCSCG-AR-ΔNLS, or pCSCG-AR-N705S lentiviruses.

siRNA Transfections. Pools of four siRNAs against AR, integrin α6, Bcl-xL, RelA, or a non-targeting sequence were purchased from Dharmacon. Cells were transfected with siRNA using siLentFect lipid reagent (Bio-Rad). Lowest concentration of siRNA able to reduce protein expression by over 85% was used.

RT-PCR. Total RNA was isolated using TRIzol and chloroform. RNA was purified with RNase-free DNase and RNeasy Mini Kits (Qiagen). RT-PCR was performed on 1µg RNA using the
One-Step RT-PCR kit (Qiagen). For qRT-PCR, 0.5µg RNA was reversed transcribed with random primers using a reverse transcription system (Promega). Synthesized cDNA was amplified for qRT-PCR using SYBR green master mix (Roche) with gene-specific primers and an ABI 7500 RT-PCR system (Applied Biosystems). Gene expression was normalized to 18S rRNA by the $2^{-\Delta\Delta Ct}$ method (25). Specific primers were as previously published: Bcl-xL, GAPDH (26), integrin α6 (27), and AR (28).

**Reporter Assays.** Laminin-adherent cells were transfected with 1.25µg pGL3-vector, pGL4.32-luc2P/NF-κB-RE, or pGL4-luc2P/ITGα6 (SwitchGear) and 0.5µg phRG-TK using Nanojuice Core Transfection Reagent and Booster Reagent (Novagen). After 48 hours, cells were lysed with Dual-Luciferase Reporter Assay System (Promega) and luminescence measured using EnVision 2104 Multilabel Reader (PERKin Elmer) and Wallac EnVision Manager Software. Firefly luminescence activity was normalized to *Renilla* luciferase activity.

**Immunoblotting.** Total cell lysates were prepared for immunoblotting as described following lysis with MAPK or RIPA buffers (8). 45-65µg of protein was run on SDS polyacrylamide gels (Invitrogen) and transferred to PVDF membranes. Membranes were blocked and processed as described (8) and visualized by chemiluminescence reagent with a CCD camera in a Bio-Rad Chemi-Doc Imaging System using Quantity One software (Bio-Rad).

**Immunofluorescence.** Cells were fixed with 4% paraformaldehyde, permeabilized with 0.2% TritonX-100, and blocked with 10% goat serum before incubation with AR (clone 411) antibody (Santa Cruz) overnight at 4°C. Cells were incubated with secondary antibody and Hoechst 33258 (Sigma), washed, and mounted using Gel-Mount (Biomeda). Epifluorescent images were acquired on a Nikon Eclipse TE300 microscope using OpenLab software (Improvision).

**FACS.** Suspended cells were washed (1% sodium azide/2% FBS/PBS) and incubated with primary antibodies or control IgG for 1 hour at 4°C and then with fluorescently-labeled secondary antibodies for 1 hour at 4°C. Fluorescence was detected by a Becton-Dickinson FACSCalibur cytometer with CellQUEST Pro Software (Becton-Dickinson).

**Antibodies.** Polyclonal antibodies to Bcl-xL, phospho-IκBα S32 (14D4), phospho-NF-κB S536 (93H1), NF-κB p65-RelA, and monoclonal antibodies to IκBα (44D4) were purchased from Cell Signaling. Polyclonal antibodies to Nkx3.1 (H-50), PSA (C-19), and monoclonal AR (411) were obtained from Santa Cruz and monoclonal anti-tubulin (DM1A) from Sigma. Integrin α6 (AA6A) was generously provided by Dr. Anne Cress and monoclonal TMPRSS2 (P5H9-A3) was
provided by Dr. Pete Nelson. Monoclonal antibodies to integrin α2 (CBL477), α3 (MAB2056), and β4 (ASC-3) were purchased from Chemicon, and α5 (P1D6) from Santa Cruz. Integrin α6 (GoH3) came from BD Pharmingen. Integrin β1 (AIIB2) monoclonal antibody, developed by Dr. Caroline Damsky (UC San Francisco, CA), was obtained from the Developmental Studies Hybridoma Bank (University of Iowa).

**Cell Survival Assays.** Laminin-adherent cells were treated with 5-20µM of LY294002 (8). In some cases, ethanol or 5-10nM each of DHT, R1881, Casodex, or RU486 was added. DHT was replenished every 24 hours. Cell viability was measured after 72 hours by collecting attached and floating cells and adding an equal volume of Trypan Blue. Three separate cell counts per well were performed on a hemocytometer; two to three wells counted per condition.

**RESULTS**

**AR promotes PI3K-independent survival.** To directly assess whether AR and integrin α6β1 cooperate to control prostate cancer survival, wild-type AR or two well-characterized AR mutants were introduced into PTEN-deficient PC3 cells. AR expression in the PC3 clones was comparable to LNCaP cells (Fig. 1A). Wild-type AR localization was both cytoplasmic and nuclear (Fig. 1B). As previously observed, the ligand-binding mutant ΔLBD (N705S), was predominately nuclear (21). The ΔNLS mutant, defective in nuclear localization (24), was exclusively cytoplasmic (Fig. 1B). PC3-AR1 and PC3-AR2 cells expressed higher levels of the AR-target genes Nkx3.1, PSA, and the activated form of TMPRSS2 (29) than the PC3-Puro control cells (Fig. 1C). Knock-down of AR in the clones reduced AR-target gene expression, indicating AR is functional. Exogenous androgen was not required for AR-target gene expression, probably because AR is already nuclear-localized in these cells (Fig. 1B).

Inhibition of PI3K with LY294002 in laminin-adherent PC3 cells induces cell death (8). To determine if AR expression could protect cells from death induced by PI3K inhibition, cells were placed on laminin (LM) in the presence or absence of LY294002. Inhibition of PI3K induced cell death in 60% of the PC3-Puro control cells (Fig. 1D). In contrast, cell death was not induced by LY294002 in the PC3-AR cells. Similar results were obtained when cell death was measured by TUNEL or propidium iodide staining (not shown). AR-dependent cell survival did not require exogenous androgen and was not observed when cells were plated on collagen, on plastic, or placed in suspension (supplementary Fig. S1), indicating this response is specific to LM. Thus, in the context of LM, AR promotes cell survival independently of PI3K.
The difference in survival was not due to cell cycle status since PC3-AR1 cells grow at the same rate, while PC3-AR2 cells grow slower, than PC3-Puro cells (not shown). Nuclear localization of AR was required for resistance to PI3K inhibition, since the AR nuclear localization defective mutant ΔNLS (Fig. 1E) was unable to confer resistance to PI3K inhibition. In contrast, the AR ligand binding mutant ΔLBD (Fig. 1F) which localizes to the nucleus (Fig. 1B) conferred resistance to PI3K inhibition. Thus, nuclear-localized AR is required to promote survival on LM independently of PI3K.

**AR promotes survival through up-regulation of laminin integrin α6β1.** FACS was used to compare integrin expression at the cell surface between PC3-AR and PC3-Puro cells. AR expression caused a 2-, 3-, and 6-fold reduction in integrin α2, α5, and α3 respectively, but increased integrin α6 levels 6-fold (Fig. 2A). There was a slight 1.5-fold decrease in integrin β1 (Fig. 2A) and a 4-fold decrease in integrin β4. Integrins are expressed as heterodimeric pairs on the cell surface and integrin α6 pairs with either β1 or β4. The corresponding decrease in the integrin β1-specific alpha subunits, i.e. α2, α3, and α5, would generate free β1 integrin making it available to bind α6. The large decrease in β4, further indicates that α6 is pairing with the β1. This predilection for integrin α6β1 mimics what is observed in prostate cancer patients (10, 11). An AR-dependent increase in integrin α6 was also observed in DU145 cells, while loss of endogenous AR in LNCaP, C4-2, or VCaP cells or in PC3-AR cells decreased integrin α6 (Fig. 2B).

The AR-dependent increase in integrin α6 expression suggested it may be responsible for the increase in survival on LM. Reduction of α6 expression by siRNA had a negligible effect on AR expression (Fig. 2C), but completely reversed the sensitivity to cell death induced by PI3K inhibition (Fig. 2D). The effect of AR on α6 was not due to a clonal artifact, as loss of AR decreased α6 expression (Fig. 2E) and restored the sensitivity to PI3K inhibition (Fig. 2F). Thus, AR promotes survival on LM independently of PI3K by increasing integrin α6 expression.

**AR stimulates integrin α6 transcription.** The AR ΔNLS nuclear localization mutant was unable to protect cells from LY294002-induced death (see Fig. 1G), suggesting AR transcriptional activity is required. Correspondingly, integrin α6 mRNA is dramatically increased in AR-expressing PC3 cells and following androgen stimulation of LNCaP or C4-2 cells (Fig. 3A,B). Reciprocally, loss of AR suppresses α6 mRNA (Fig. 3C). Furthermore, cells expressing the ΔNLS mutant failed to up-regulate α6 (Fig. 3D), and the AR transcriptional repressors Casodex and RU486 (30) decreased integrin α6 mRNA (Fig. 3E) and protein (not shown). Casodex also restored the sensitivity to cell death induced upon PI3K inhibition (Fig. 3F). Thus,
the transcriptional activity of AR is required to increase integrin α6 expression and confer resistance to cell death.

R1881-induced integrin α6 mRNA was observed as early at 6 hours (Fig. 3G) and peaked at 8-12 hours (Fig. 3H). Induction of α6 mRNA was resistant to cycloheximide treatment, indicating the synthesis of other proteins is not required. Interestingly, combined R1881 and cycloheximide treatment enhanced α6 transcription suggesting the presence of a protein synthesis-sensitive α6 repressor which is blocked in response to androgen. Expression of a luciferase reporter containing ~1kb of the α6 promoter was elevated in PC3-AR cells relative to PC3-Puro cells (Fig 3I) and stimulated by R1881 in LNCaP cells (Fig. 3J). These data indicate AR directly stimulates integrin α6 transcription.

**Bcl-xL is required for AR/α6β1-dependent survival.** We previously demonstrated that adhesion to LM increases Bcl-xL expression (8). Therefore, we postulated the AR-mediated increase in integrin α6 should increase Bcl-xL expression. Bcl-xL was dramatically up-regulated in PC3-AR cells, and loss of α6 by siRNA decreased Bcl-xL while loss of AR decreased both α6 and Bcl-xL expression (Fig. 4A). Bcl-xL mRNA was also increased by AR (Fig. 4B). Stimulation of LNCaP, C4-2, or VCaP cells (Fig. 4C-D, 3H) with androgen or knock-down of AR (Fig. 4E) correspondingly altered α6 and Bcl-xL mRNA. Thus, AR stimulation of integrin α6 expression leads to increased Bcl-xL mRNA and protein expression.

Reduced Bcl-xL expression in PC3-AR cells by siRNA (Fig. 4F) restored the sensitivity to death induced by PI3K inhibition (Fig. 4G). Complete loss of Bcl-xL resulted in complete loss of viability of both PC3-Puro and PC3-AR cells (not shown). Conversely, over expression of Bcl-xL in parental PC3 cells, to the levels seen in PC3-AR cells (Fig. 4H), was sufficient to confer resistance to PI3K inhibition (Fig. 4I). Thus, Bcl-xL promotes survival of LM-adherent prostate cancer cells independent of PI3K.

**NF-κB signaling is required for PI3K-independent survival.** Our data indicate that AR controls Bcl-xL expression indirectly through integrin α6 (see Fig. 4A,B). NF-κB has been reported to bind directly to the Bcl-xL promoter and drive its transcription, and α6 has been shown to regulate NF-κB (31-33). NF-κB p65-RelA activity was increased in PC3-AR cells (Fig 5A-B) and inhibited upon AR knock-down in C4-2, VCaP, or PC3-AR cells (Fig 5C-F). Conversely, NF-κB-RelA activity was increased upon androgen-stimulation and its activity paralleled the increase in α6 and Bcl-xL expression, peaking at 24 hours (Fig 5D-E). Increased phosphorylation of both IKKβ and IκBα was also observed (Fig. 5G). Knock-down of integrin α6
in PC3-AR, C4-2, or LNCaP cells decreased RelA phosphorylation and Bcl-xL expression (Fig. 6A-B). Knock-down of RelA resulted in a partial loss of Bcl-xL (Fig. 6B-C), but was sufficient to sensitize C4-2 and PC3-AR cells to LY294002-induced death (Fig. 6D-E). Furthermore, the ability of androgen to rescue LNCaP or C4-2 cell death induced by PI3K inhibition, as previously reported (18, 19), is abrogated when AR, α6, or RelA expression is suppressed (Fig. 6F,G). Thus, NF-κB-RelA activity is increased in an AR- and integrin α6-dependent manner, and in part controls Bcl-xL expression downstream of integrin α6. This pathway is responsible for conferring resistance to death induced by PI3K inhibition when cells are adherent to LM.

DISCUSSION

In this study, we identified an AR-dependent prostate cancer cell survival pathway that operates independently of PI3K when tumor cells are adherent to LM. Resistance to death-induced by PI3K inhibition is mediated via AR-dependent transcriptional stimulation of integrin α6 mRNA lead to increased α6β1 cell surface expression. Integrin α6β1 engagement of LM subsequently activates NF-κB and increases Bcl-xL expression (Fig. 7). Down-regulation of AR, integrin α6, NF-κB, or Bcl-xL re-sensitizes AR-expressing cells to PI3K-dependent survival.

Previous studies, in which AR was re-expressed in prostate tumor cell lines, reported reduced proliferation or cell survival due to activated AR (34-36). Therefore, extra precautions were taken to keep AR minimally active in our cells. First, the AR cDNA was sequence-verified to be wild-type and not an activated variant. Second, AR was not highly over-expressed, but maintained at levels similar to LNCaP cells. Third, only low passage (<20) cells were used, since phenotypes can change with passage. Fourth, cells were isolated and constantly maintained in charcoal-stripped serum and phenol red-reduced media to prevent over activation of AR. Immunostaining indicates that even under these conditions a large portion of AR is nuclear-localized in the absence of exogenous ligand. It is possible the constitutive nuclear localization of AR in our cells is a reflection of the known steroidogenic activity present in PC3 cells resulting in intracellular synthesis of androgen (37-39). This could explain why addition of exogenous androgen to PC3-AR cells does not enhance AR function. Furthermore, continual addition of exogenous androgens in this system, such as propagation of cells in non-stripped serum, could hyper-activate AR such that it acts a suppressor and thus explain why it might suppressed growth and reduced survival as seen by others (40).

Loss of responsiveness to exogenous androgens in AR-expressing cells, in which AR is still active due to synthesis of intracellular androgens, is characteristic of castration-resistant
tumors. Thus, the PC3-AR model may reflect events associated with castration-resistant cancers. In support of this, previous studies have linked increased NF-κB activity with prostate cancer progression and metastasis (41, 42), castration-resistance (43, 44), poor prognosis (45), and biochemical failure (i.e., PSA relapse) (46). Similarly, increased Bcl-xL expression is associated with prostate cancer progression and castration-resistance (26, 47, 48).

Furthermore, we observed that androgen-sensitive LNCaP cells have significantly less integrin α6 and Bcl-xL expression than the castration-resistant derived C4-2 subline. Our study indicates that AR is responsible for the increase in NF-κB activation as reported by others (42, 43), that this is mediated by AR-dependent stimulation of integrin α6β1 expression, and that LM-mediated activation of NF-κB contributes to Bcl-xL expression.

Oddly, while NF-κB or Bcl-xL knock-down was sufficient to completely re-sensitize cells to death induced by PI3K inhibition, NF-κB knock-down, unlike AR or integrin α6 loss, resulted in only a partial loss of Bcl-xL. The partial knock-down of Bcl-xL by NF-κB loss may be sufficient for AR expressing cells to regain dependence on PI3K signaling. Alternatively, NF-κB may regulate other cell survival molecules whose loss upon inhibition of NF-κB contributes to this phenotype.

Our finding that AR increases integrin α6 expression is consistent with the observation that constitutive AR expression in immortalized prostate epithelial cells leads to increased α6 (13) and its singular expression in prostate cancer tissues and metastases (11, 12). However, previous AR re-expression studies in PC3 or DU145 cells did not report an increase in integrin α6 expression (34, 35, 49). Possible explanations include differences in the level of AR re-expression, use of non-charcoal stripped serum for cultivation, duration of growth-factor and serum starvation prior to experimental assays, and passage number used. However, the most significant difference was that the integrin expression assays in the other studies were done with cells plated on plastic, while in our studies cells were adherent to LM. Adhesion to LM may result in increased integrin α6 stabilization, explaining this observed difference. Nonetheless, AR is still required in this context to control α6 expression. It is possible that in prostate cancer, elevated integrin α6β1 expression is also dependent on engagement of the integrin by LM. The preferred ligands for α6β1 are LM10 and LM1. LM10 is the expressed in adult tissues, while LM1 is predominantly embryonic. LM10 is present in prostate tumors and bone metastases. Due to lack of availability of purified LM10, we used LM1 in our studies. We assume similar signaling pathways are activated on the two matrices, but it is possible there could be some differences.
The full-range of transcriptional mechanisms that control integrin α6 expression has not been extensively studied. AR appears to directly regulate α6 transcription, since the response occurs within 6 hours and is not blocked by cycloheximide. In addition, the first kilobase of the α6 promoter is sufficient for activation by AR. However, this region does not contain canonical AR response elements (14, 50). Progesterone, but not estradiol, can increase α6 promoter activity via an imperfect steroid response element in this region (14). Our preliminary studies suggest that AR binds to a region containing this steroid response element.

Detection of the AR/α6β1 survival pathway requires that the constitutive PI3K signaling, due to PTEN loss, be simultaneously inhibited. Previous studies in PTEN-negative LNCaP cells suggested that survival of castration-resistant variants was mediated by augmenting PI3K signaling (51). We failed to detect an increase in PI3K signaling, as measured by Akt, BAD, survivin, or FOXO activation, above that seen in the vector control cells and LY294002 alone failed to induce any death above basal levels. It is possible that upon adhesion to LM, the AR/α6β1 pathway precludes the need for survival signaling through PI3K. Inhibition of Src kinases also induces the death of LM-adherent PC3 cells (8). In addition to being resistant to PI3K inhibition, PC3-AR cells are also resistant to inhibition of Src kinases (Supplemental Fig. S2), but are not resistant to death induced by TNFα or staurosporine. Thus, other pathways may also be involved in controlling prostate tumor cell survival.

Interestingly, integrin α2β1, which mediates adhesion to collagen, was only slightly decreased in the PC3-AR cells, and when plated on collagen, both the control and PC3-AR lines were resistant to PI3K inhibition. These data indicate that integrin α2β1 also controls PC3 survival independent of PI3K, but also independently of AR. The differences in survival mechanisms on specific matrices suggest that, depending on the tumor microenvironment, different integrins may activate distinct signaling pathways to promote survival. These data have important therapeutic implications for treatment, whereby both AR/α6β1 and PI3K signaling may need to be targeted to efficiently kill prostate cancer cells adherent to LM. On the other hand, if collagen is present, another pathway may be able to compensate.

In summary, we have identified an AR-dependent pathway acting through α6β1 that stimulates survival of LM-adherent prostate cancer cells independently of PI3K signaling. AR/α6β1 stimulates the activity of NF-κB and Bcl-xL, whose up-regulation is highly associated with advanced hormone-refractory prostate cancer. Application of this new knowledge may lead to the development of better prostate cancer therapies, and supports the importance of targeting more than one pathway to effectively treat prostate cancer.
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REFERENCES


FIGURE LEGENDS

FIGURE 1. AR stimulates cell survival independently of PI3K. A) AR and tubulin (Tub) expression in LNCaP, PC3 vector controls (Puro or pLKO), and PC3 cells expressing wild-type AR (AR), a ligand-binding mutant (ΔLBD), or a nuclear-localization mutant (ΔNLS) monitored by immunoblotting. B) PC3-AR1 (AR), ΔLBD28 (ΔLBD), and ΔNLS4 (ΔNLS) cells immunostained for AR (green) and counterstained for DNA (red). Yellow indicates co-localization. C) Nkx3.1 (Nkx), TMPRSS2 (TMP), PSA, AR, and tubulin (Tub) expression in PC3-Puro, AR1, and AR2 cells treated with vehicle (-) or 10nM DHT (+) or treated with AR (si) or control (scr) siRNA. D) Viability of PC3-Puro, AR1, AR2 or E-F) PC3-pLKO, ΔNLS, or ΔLBD cells plated on LM, treated with vehicle (-) or 10nM DHT in the presence of DMSO or LY294002 (LY). Error bars are S.D.; n = 3-5.

FIGURE 2. AR promotes survival through up-regulation of integrin α6β1. A) FACS analysis of integrin expression in LM-adherent PC3-Puro (PP) (solid black), AR1 (solid dark grey), and AR2 (solid light grey) cells. IgG control is dashed line. Small arrows indicate direction of peak shifts. n = 5-8. B) AR, integrin α6 (ITGα6), and tubulin expression in PC3-AR, LNCaP, C4-2, or VCaP cells treated with AR (siAR) or control (scr) siRNA or in DU145 clones expressing AR monitored by immunoblotting. C-F) PC3-Puro, AR1, and AR2 cells treated with integrin α6 (siα6), AR, or control siRNA. C,E) Integrin α6, AR, and tubulin immunoblots. D,F) Viability after DMSO or LY294002 (LY) treatment.

FIGURE 3. AR transcriptionally regulates integrin α6β1. A) Integrin α6 (ITGα6) mRNA measured by qRT-PCR in PC3-Puro, AR1, and AR2 cells. B-C) Integrin α6 or AR mRNA measured by qRT-PCR in B) LNCaP and C4-2 or C) LNCaP cells treated 24 hours with vehicle (Veh) or 5nM R1881 or C) 48 hours with AR or control siRNA. D) FACS analysis of α6 expression in AR1, AR2, ΔNLS-AR4, and ΔNLS-AR30 cells. Values are normalized to vector control cells. E) Integrin α6 mRNA measured by qRT-PCR in PC3-AR1 cells treated with vehicle (EtOH), 10nM Casodex (Caso), or 10nM RU486 (RU). F) Viability of PC3-AR1 or AR2 cells treated with Casodex in the absence or presence of LY294002 (LY). G) Time course of α6 and GAPDH mRNA in VCaP cells stimulated with 5nM R1881 (R) in the absence or presence of 10µg/ml cycloheximide (Cx). H) Time course of PSA, α6, Bcl-xL and GAPDH mRNA in C4-2 cells stimulated with 5nM R1881. I) Luciferase activity in PC3-Puro, PC3-AR1, or J) LNCaP cells transiently transfected with vector (pGL3-vec) or integrin α6 reporters (pITGα6). LNCaPs were treated with vehicle (veh) or 5nM R1881 for 24 hours.
FIGURE 4. Bcl-xL promotes AR/α6β1-dependent survival independent of PI3K.  A) AR, α6, Bcl-xL, and tubulin expression in PC3-Puro, AR1, and AR2 cells treated with α6, AR, or control siRNA monitored by immunoblotting.  B-E) Bcl-xL or α6 mRNA measured by qRT-PCR in B) PC3-Puro, AR1, AR2, C,E) LNCaP, C4-2, or D) VCaP cells treated with C,D) R1881 or E) siRNA.  F-G) Cells treated with Bcl-xL (si-xL) or control siRNA.  F) Bcl-xL, AR, and tubulin immunoblots.  G) Viability of DMSO- or LY294002-treated cells.  H) Bcl-xL, AR, and tubulin expression in PC3 cells stably over-expressing Bcl-xL.  I) Viability of PC3-Puro and Bcl-xL (Bxl) clones treated with DMSO or LY294002 (LY).

FIGURE 5. AR stimulates NF-kB activity.  A-B,F) PC3-Puro (PP), AR1, and AR2 cells, or C-E) C4-2 and VCaP cells treated with AR or control siRNA, or treated with vehicle or R1881.  NF-kB activity measured by A,D-F) immunoblotting for phosphorylated RelA (pRelA) or B,C) transfection of an NF-kB luciferase reporter.  Integrin α6, Bcl-xL, or total RelA measured by immunoblotting.  F) Control cells not treated (NT) or treated with 10ng/mL TNFα for 1 hour.  G) IKKβ (pIKKβ) and IκBα (pIκBα) phosphorylation monitored by immunoblotting of immunoprecipitated IKKβ or IκBα in total cell lysates with phospho-specific antibodies.

FIGURE 6. Integrin α6 stimulates NF-kB activity and survival.  A-C) PC3-Puro, AR1, AR2, LNCaP, or C4-2 cells treated with α6, RelA (siRel), or control siRNA.  Control cells not treated (NT) or treated with 10ng/mL TNFα.  RelA phosphorylation, total RelA, AR, α6, Bcl-xL, or tubulin monitored by immunoblotting.  D-E) Viability of RelA siRNA-transfected C4-2, PC3-Puro, AR1, or AR2 cells treated with DMSO or LY294002 (LY).  F-G) Viability of LNCaP or C4-2 cells transfected with control, AR, α6, or RelA siRNA and subsequently treated with DMSO, LY294002, or LY294002 + R1881.

FIGURE 7. Model for AR/α6β1-mediated survival.  AR stimulates integrin α6 transcription and expression leading to canonical activation of NF-kB and up-regulation of Bcl-xL.  NF-kB, in part, increases Bcl-xL expression.  NF-kB and Bcl-xL are required for survival on laminin independent of PI3K.
FIGURE 1
FIGURE 3

A. ITGα6

Fold mRNA

B. ITGα6

Level mRNA

C. ITGα6

Fold mRNA

D. FACS - ITGα6

Fold Increase

E. Level mRNA

F. Cell Death

G. VCaP

ITGα6 -

GAPDH -

H. R1881: 0 8 24 31 42 52 69 hr

PSA -

ITGα6 -

Bcl-xL -

GDH -

I. LNCaP

Luciferase Units

J. pITGα6

Luciferase Units

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FIGURE 4

A

B

C

D

E

F

G

H

I

Level mRNA

Fold mRNA

Bcl-xL

Bcl-xL

Bcl-xL

Bcl-xL

Bcl-xL

Bcl-xL

Bcl-xL
FIGURE 5
The Androgen Receptor Induces Integrin α6β1 to Promote Prostate Tumor Cell Survival via NF-κB and Bcl-xL Independently of PI3K Signaling

Laura E Lamb, Jelani C Zarif and Cindy K Miranti

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