Biochemical and Biophysical Research Communications 476 (2016) 69–74

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Restoration of the cellular secretory milieu overrides androgen dependence of *in vivo* generated castration resistant prostate cancer cells overexpressing the androgen receptor

Mugdha Patki, Yanfang Huang, Manohar Ratnam*

Barbara Ann Karmanos Cancer Institute, Department of Oncology, Wayne State University, Detroit, MI 48201-2013, USA

ARTICLE INFO

Article history:
Received 6 May 2016
Accepted 11 May 2016
Available online 12 May 2016

Keywords:
Androgen
Androgen receptor
Androgen deprivation therapy
Castration resistant prostate cancer

ABSTRACT

It is believed that growth of castration resistant prostate cancer (CRPC) cells is enabled by sensitization to minimal residual post-castrate androgen due to overexpression of the androgen receptor (AR). Evidence is derived from androgen-induced colony formation in the absence of cell-secreted factors or from studies involving forced AR overexpression in hormone-dependent cells. On the other hand, standard cell line models established from CRPC patient tumors (e.g., LNCaP and VCaP) are hormone-dependent and require selection pressure in castrated mice to re-emerge as CRPC cells and the resulting tumors then tend to be insensitive to the androgen antagonist enzalutamide. Therefore, we examined established CRPC model cells produced by castration of mice bearing hormone-dependent cell line xenografts including CRPC cells overexpressing full-length AR (C4-2) or co-expressing wtAR and splice-variant AR-V7 that is incapable of ligand binding (22Rv1). In standard colony formation assays, C4-2 cells were shown to be androgen-dependent and sensitive to enzalutamide whereas 22Rv1 cells were incapable of colony formation under identical conditions. However, both C4-2 and 22Rv1 cells formed colonies in conditioned media derived from the same cells or from HEK293 fibroblasts that were proven to lack androgenic activity. This effect was (i) not enhanced by androgen, (ii) insensitive to enzalutamide, (iii) dependent on AR (in C4-2) and on AR-V7 and wtAR (in 22Rv1) and (iv) sensitive to inhibitors of several signaling pathways, similar to androgen-stimulation. Therefore, during progression to CRPC *in vivo*, coordinate cellular changes accompanying overexpression of AR may enable cooperation between hormone-independent activity of AR and actions of cellular secretory factors to completely override androgen-dependence and sensitivity to drugs targeting hormonal factors.

1. Introduction

Growth of castration resistant prostate cancer (CRPC) may often be supported by restoration of tumor androgen to levels approaching or greater than pre-castration levels via *de novo* intratumoral synthesis [1,2]. It has also been proposed, based on *in vitro* studies, that overexpression of AR, a common feature of clinical CRPC, sensitizes CRPC cells to much lower, castrate levels of androgen [3–5]. In such cells, *in vitro* data also suggests that low androgen may synergize with hyperactive cell signaling pathways to support optimal tumor growth [6]. Overexpression of AR splice variants with deletions spanning the ligand-binding pocket have also been functionally and clinically linked to prostate cancer progression; in these cases expression of full-length AR may also be necessary [7]. Nevertheless, in patients with CRPC, treatment with the high affinity AR antagonist enzalutamide or the androgen synthesis inhibitor abiraterone has produced limited clinical benefit [8,9]. Efforts are therefore underway to develop more effective inhibitors of residual androgen biosynthesis as well as higher affinity androgen antagonists to treat CRPC [10,11]. In this context, the following considerations warrant further investigation of whether AR-overexpressing CRPC cells that develop under castrate conditions are necessarily androgen-dependent in the physiological secretory microenvironment of the cells.

First, the *in vitro* studies demonstrating androgen-dependence of AR-overexpressing CRPC cells often relied on colony formation assays in media containing charcoal-stripped serum. These assay conditions are hence devoid of many serum factors and adequate levels of factors secreted by neighboring cells because of the

* Corresponding author. 4100 John R, HWCRC 840.1, Detroit, MI 48201, USA.
E-mail address: ratnamm@karmanos.org (M. Ratnam).

http://dx.doi.org/10.1016/j.bbrc.2016.05.058
0006-291X/

extremely low density of cells in colony formation assays. Although AR-overexpressing CRPC cells generated in castrated mice (e.g., C4-2 cells) require androgen in such colony formation assays, they grow robustly in monolayer culture in media containing charcoal-stripped serum and are also insensitive to enzalutamide in monolayer cultures [12]. In fact, sensitivity of monolayer cultures and tumor xenografts to enzalutamide is typically demonstrated using CRPC model cells produced by forced overexpression of AR in hormone-sensitive cell lines to render them hyper-sensitive to androgen [11,13,14]. Another CRPC model used to demonstrate androgen dependence comprises AR + androgen-dependent cells with forced expression of an AR splice variant incapable of ligand binding [15]. Second, standard cell line models established from metastatic CRPC patient tumors (e.g., LNCaP and VCaP) are hormone-dependent and require selection pressure in castrated mice to re-emerge as CRPC cells and the resulting tumors then tend to be insensitive to the high affinity androgen antagonist enzalutamide [12]. This suggests that *in vivo* generated CRPC tumors may be androgen-independent in contrast to CRPC model cells generated by forced AR overexpression. Third, studies demonstrating the need for androgen to potentiate growth signaling by autocrine/paracrine pathways in AR-overexpressing CRPC cells were focused on activation of individual signaling pathways rather than collective activation of multiple cellular events that might render the cells completely hormone-independent.

Therefore, we hypothesized that progression to CRPC *in vivo*, associated with overexpression of endogenous wtAR, could include cellular changes that enable the complement of cell-secreted factors to support and/or cooperate with hormone-independent AR actions; this would override the androgen-dependence observed in the absence of such factors. Further, overexpressed AR splice variants in CRPC cells may similarly need to cooperate with cell-secreted factors to support hormone-independent CRPC growth. We tested this hypothesis using the *in vivo* generated C4-2 CRPC cells that overexpress full length AR. In parallel, we examined the *in vivo* generated 22Rv1 CRPC cells, which overexpress a common AR splice variant (AR-V7) in addition to expressing wtAR. C4-2 cells and 22Rv1 cells were originally produced in mice via castration-induced selection pressure placed on the hormone-dependent LNCaP and CWR22 cell lines, respectively.

2. Materials and methods

VCaP cells, 22Rv1 cells were from the American Type Culture Collection (Manassas, VA). C4-2 cells were kindly provided by Dr. Edwin Sanchez (University of Toledo). All cell lines were routinely maintained as previously described [16].

To prepare conditioned media, C4-2 cells, 22Rv1 cells and HEK 293 fibroblasts were grown in phenol-red free RPMI 1640 medium supplemented with 10% heat-inactivated charcoal stripped FBS for two passages. Forty-eight hour conditioned media was then collected, centrifuged and filtered through a 0.2 μ filter.

Colony formation assays, cell proliferation assays and western blots were performed as previously described [16].

RNA isolation, reverse transcription and qPCR methods have been described [16].

Lentivirus-mediated gene knockdown has been previously described [16]. (shRNA sequences provided in Supplementary Table 1).

All experiments were repeated at least three times. Statistical significance was determined using one-way analysis of variance.

3. Results

In media containing charcoal-stripped serum, androgen (R1881) was obligatory for colony formation of C4-2 cells (Fig. 1A) whereas 22Rv1 cells failed to form colonies either in the absence or in the presence of R1881 (Fig. 1B). Both C4-2 cells (Fig. 1A) and 22Rv1 cells (Fig. 1B) showed robust colony formation in 48-h conditioned media from either the same cells or from HEK293 fibroblasts grown in media containing charcoal-stripped serum. The addition of R1881 to either type of conditioned media did not increase the number of colonies (Fig. 1A and B).

As structurally diverse molecules could have androgenic activity, we first used a sensitive biological test to detect any androgenic activity in the conditioned media used above. For this purpose, we used VCaP cells, which are exquisitely dependent on androgen for both survival and growth. In media containing charcoal-stripped serum, survival of VCaP cells at the end of 4 days was reduced by ~50% (Fig. 1C). The cells required at least 0.025 nM R1881 to prevent this loss of viability and higher concentrations of R1881 were required for cell growth (Fig. 1C). However, survival of VCaP cells in conditioned media from C4-2, 22Rv1 or HEK293 cells was similar to that in the absence of R1881. From inspection of Fig. 1C, it appears that any androgenic activity present in the conditioned media must be less than the equivalent of 0.003 nM R1881. Since, under androgen-dependent conditions, colony formation of C4-2 cells required 0.05 nM R1881 for optimal colony formation (Fig. 1D), it is evident that the conditioned media did not have the level of androgenic activity required to support colony formation.

As another test for androgenic activity in the conditioned media we examined inhibition of colony formation by the high affinity androgen antagonist, enzalutamide. The androgen-dependent colony formation of C4-2 cells (in 0.1 nM R1881) was completely blocked by enzalutamide at concentrations of 1 μM and 10 μM but not at 0.1 μM enzalutamide (Fig. 2A). The enzalutamide dose-dependence for inhibition of colony formation in C4-2 cells corresponded to the dose required to completely antagonize gene activation by R1881 (0.1 nM) in the same cells as measured for the KLK3 and TMPRSS2 genes (Fig. 2B and C). Enzalutamide did not inhibit colony formation of C4-2 cells in conditioned media from the same cells or HEK293 fibroblasts (Fig. 2A). Similar to C4-2 cells, enzalutamide did not inhibit colony formation of 22Rv1 cells in conditioned media from the same cells or HEK293 fibroblasts (Fig. 2D). These results further confirm lack of androgenic activity in the conditioned media from C4-2, 22Rv1 and HEK293 cells.

Depleting AR by lentiviral transduction of targeted shRNA abrogated the ability of conditioned media from C4-2 or HEK293 cells to support colony formation in C4-2 cells (Fig. 2E). In 22Rv1 cells, knockdown of full-length AR alone or a partial knockdown of total AR (full length AR plus AR-V7) abrogated colony formation in conditioned media from 22Rv1 or HEK293 fibroblasts (Fig. 2F). The results demonstrate that despite the absence of androgenic activity, the ability of conditioned media to support colony formation is dependent on expression of AR in both C4-2 and 22Rv1 cells. Further, it may be noted (from the combined data in Figs. 1B, 2D and F) that, in 22Rv1 cells the full length AR is required to support colony formation but this occurs in an androgen-independent manner.

We used a battery of signaling pathway inhibitors (described in Supplementary Table 2) to compare androgen-dependent colony formation in C4-2 cells and the androgen-independent colony formation of C4-2 and 22Rv1 cells in various conditioned media. The androgen-dependent colony formation of C4-2 cells was prevented by inhibitors of Akt, PI3-K, EGF/FGF/PDGF receptor tyrosine kinase, Rac1 and STAT3 but not by inhibitors of FGFR, VEGFR, Src family, c-Raf, MEK1/2, ERK, JNK and PKC (Fig. 3A). The pattern of inhibitor sensitivity of colony formation of C4-2 cells in conditioned media from C4-2 cells or HEK293 fibroblasts was virtually identical to that for androgen-dependent colony formation (Fig. 3B). Colony