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Mechanisms Mediating Androgen Receptor Reactivation After Castration

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SUMMARY

Androgen deprivation is still the standard systemic therapy for metastatic prostate cancer (PCa), but patients invariably relapse with a more aggressive form of PCa termed hormone refractory, androgen independent, or castration resistant PCa (CRPC). Significantly, the androgen receptor (AR) is expressed at high levels in most cases of CRPC, and these tumors resume their expression of multiple AR-regulated genes, indicating that AR transcriptional activity becomes reactivated at this stage of the disease. The molecular basis for this AR reactivation remains unclear, but possible mechanisms include increased AR expression, AR mutations that enhance activation by weak androgens and AR antagonists, increased expression of transcriptional coactivator proteins, and activation of signal transduction pathways that can enhance AR responses to low levels of androgens. Recent data indicate that CRPC cells may also carry out intracellular synthesis of testosterone and DHT from weak adrenal androgens and may be able to synthesize androgens from cholesterol. These mechanisms that appear to contribute to AR reactivation after castration are further outlined in this review.

Keywords

androgen receptor; prostate cancer; testosterone; androgen; androgen deprivation therapy; AR antagonist

The androgen receptor (AR) is a steroid receptor member of the larger nuclear receptor superfamily, and plays a central role in normal prostate development and in prostate cancer (PCa) initiation and progression. In the absence of androgens (testosterone or dihydrotestosterone, DHT) the AR is inactive and associates with an Hsp90 chaperone complex. Androgen binding results in conformational changes that lead to dimerization, binding to androgen responsive elements (AREs) in androgen regulated genes, and increased transcription of these AR target genes (Fig. 1). The major role of AR in normal prostate is to drive differentiation of luminal epithelial cells and regulate the transcription of protein products that are required for prostate function, such prostate specific antigen (PSA). The critical function or functions of AR in PCa have been less clear, but are presumably to stimulate the expression of a series of genes that regulate cell cycle and are required for PCa survival or growth [1,2]. A recent major breakthrough was the discovery of gene fusions

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between Ets transcription factors (primarily *ERG*) and the strongly androgen regulated *TMPRSS2* gene in a large fraction of primary PCa and in associated PIN lesions [3–5]. This gene fusion is presumed to result in AR stimulated overexpression of Ets transcription factors, which may then mediate or strongly contribute to PCa development (Fig. 1).

Androgen deprivation therapy (ADT) is still the standard systemic treatment used for locally advanced or metastatic PCa. Approximately 80% of patients treated with androgen deprivation therapies, which suppress testicular androgen production (surgical castration or administration of LHRH superagonists) or block AR by treatment with AR antagonists (flutamide or bicalutamide), show clinical and biochemical (decrease in serum PSA) evidence of improvement. Unfortunately, patients invariably relapse with a more aggressive form of PCa that has been termed hormone refractory, androgen independent, or castration resistant PCa (CRPC). Significantly, multiple immunohistochemical studies have shown that AR protein is expressed at high levels (comparable to levels in untreated tumors) in most cases of CRPC, although the expression may be heterogeneous with a fraction of cells in some tumors being AR low or negative [6–8]. Consistent with the immunohistochemical results, AR mRNA is also highly expressed in CRPC, with levels being several fold higher than in primary untreated tumors [9–11]. At least one mechanism for the increased AR mRNA expression is AR gene amplification, which occurs in about one-third of CRPC cases [12,13].

In addition to expressing AR, these CRPC resume their expression of multiple AR regulated genes (such as *PSA* and including *TMPRSS2:ERG* fusion genes), indicating that AR transcriptional activity becomes reactivated at this stage of the disease [8,10,11,14,15]. Studies using PCa cell lines and xenografts similarly show that progression to CRPC is associated with high levels of AR and resumed expression of androgen regulated genes [16–22]. Moreover, AR downregulation in cell lines at this CRPC stage (by siRNA or other methods) can suppress tumor growth, indicating that AR continues to provide critical functions [17,22–24].

Although these data indicate that AR transcriptional activity is reactivated in CRPC, the molecular basis for this reactivation remains unclear and multiple mechanisms are likely to be involved (Fig. 1). Increased AR expression may enhance any weak residual AR activity that remains after castration, and can enhance growth of a PCa cell line in castrated mice [17]. Several groups have identified *AR* mutations in CRPC that can enhance AR activation by weak adrenal androgens and other steroid hormones such as progesterone, estradiol, and cortisol. These mutations may also alter AR responses to certain antagonists such as hydroxyflutamide (the active metabolite of flutamide) and bicalutamide, so that these drugs act as potent agonists [9,25–29].

However, the overall frequency of *AR* mutations in patients treated initially with surgical castration or LHRH agonists (androgen deprivation monotherapy) is quite low, and this is unlikely to be a major mechanism for progression to CRPC [30]. Nonetheless, mutant ARs that are stimulated by the AR antagonist flutamide are much more frequent in patients treated long term with this drug in combination with castration as their initial hormonal therapy (combined androgen blockade). Moreover, these patients also have increased responses to another AR antagonist (bicalutamide) that can still block the mutant ARs [26,31,32]. Finally, the *AR* mutation in codon 741 that allows bicalutamide to function as an agonist has been found in patients treated with bicalutamide, and in LNCaP cells after long term culture with bicalutamide [30,33,34]. Taken together, these findings demonstrate that AR antagonists can generate strong selective pressure for mutations that enhance AR activity, but that alternative mechanisms are responsible for AR reactivation in most patients.

While castration reduces the levels of circulating testosterone and DHT, there are still substantial levels of these hormones in serum and in tissues (see below), and there are multiple mechanisms by which tumors with wild-type AR may adapt and respond to these decreased androgen levels. One general mechanism is by increased expression of transcriptional coactivator proteins such as SRC-1 and SRC-2 (TIF2), which can enhance AR transcriptional responses to low levels of androgen [35]. A second general mechanism is by activation of certain kinases or kinase signal transduction pathways. These include protein kinase A, Cdk1, PI3 kinase/Akt, and the Ras-Raf-MAP kinase pathway that may be activated by upstream receptor tyrosine kinases. Studies from many groups have shown that activation of these kinase pathways in PCa cell lines or in AR transfected cells can enhance AR activation in response to low levels of androgen, with the dose-response curve to androgens shifting by one hundred to one thousand fold lower androgen concentrations in some cases [20,35–46]. Some studies also suggest that the AR may be activated in the total absence of ligand, but it is not clear that very low levels of residual ligands can be excluded. Importantly, activation of the PI3 kinase/Akt and Ras-Raf-MAP kinase pathway, and increased expression of receptor tyrosine kinases such as HER2/Neu/ErbB2 are observed in more aggressive primary PCa and in CRPC [20,22,40,47]. Therefore, it appears likely that these pathways do contribute to the generation of an AR that is “hypersensitive” to low levels of androgen, and that this is an important mechanism for PCa progression after castration.

While these kinase pathways can enhance AR activity, the effects in general appear to be indirect and mediated through phosphorylation of coactivator proteins, rather than through direct AR phosphorylation [43]. Previous studies have established that the AR is phosphorylated at multiple serine/threonine sites, but the specific kinases mediating phosphorylation at each site and the functional importance of these sites are not clear [48–51,51]. In the case of Cdk1, which enhances AR activity and can directly phosphorylate AR, the effects of Cdk1 are not abrogated by mutation of individual Cdk1 target sites (indicating that either multiple sites are involved or that Cdk1 is modulating AR indirectly through phosphorylation of other proteins) [45]. However, an exciting recent advance has been the finding that AR can be phosphorylated, at least transiently, at several tyrosine residues. This tyrosine phosphorylation can be mediated by at least two tyrosine kinases, Src and Ack1, and enhances AR responses to low levels of androgen [52–54].

A further mechanism that may contribute to AR activation after castration is increased androgen synthesis by the tumor cells (Fig. 2). Previous studies have established that stromal cells in normal prostate express the enzyme that can reduce androstenedione to testosterone, aldo-keto reductase family 1, member C3 (AKR1C3, also referred to as 17 β -hydroxysteroid dehydrogenase type 5, 17 β HSD5; or 3 α -hydroxysteroid dehydrogenase type 2) [55–59]. Interestingly, testosterone synthesis in Leydig cells in the testes is mediated by a distinct enzyme, type 3 17 β -HSD [60]. Prostate epithelium also expresses the type 1 and type 2 5 α -reductases that reduce testosterone to the higher affinity ligand DHT, with the type 2 5 α -reductase being the predominant isoform in normal prostate. The function of these enzymes is presumably to buffer the prostate against fluctuations in serum androgen levels and insure that the AR remains constitutively liganded. Consistent with this intraprostatic synthesis of testosterone and DHT, healthy men treated with a GnRH antagonist had a 94% decline in serum testosterone, but only a 70–80% decline in prostate tissue levels of testosterone and DHT [61]. Other studies in healthy men or patients undergoing neoadjuvant hormone deprivation therapy prior to radical prostatectomy have similarly shown that intraprostatic androgen levels do not decline as markedly as systemic levels [62–65].

In a study of gene expression in CRPC bone metastases versus laser capture microdissected primary “androgen dependent” PCa, we found that AKR1C3 expression was increased ~5-

fold in the CRPC samples [11]. This study also found increased expression of the type 1 5 α -reductase and reduced expression of the type 2 5 α -reductase, which has been confirmed in other studies [66]. Expression of the *AKR1C3* related genes, *AKR1C2* and *AKR1C1*, was also increased in the CRPC tumors. *AKR1C2* reduces DHT to the less active 5 α -androstan-3 α ,17 β -diol (3 α -diol), which is subsequently glucuronidated to 5 α -androstan-3 α ,17 β -diol glucuronide (3 α -diolG) [58,67,68]. *AKR1C1* reduces DHT to 5 α -androstan-3 β ,17 β -diol (3 β -diol), a possible endogenous ligand for the estrogen receptor β [69,70]. Finally, there was increased expression of UDP glycosyltransferase 2, B15 (*UGT2B15*), which in conjunction with *UGT2B17* mediates the glucuronidation of DHT metabolites. This increase in genes mediating DHT catabolism (*AKR1C2*, *AKR1C1*, and *UGT2B15*) may be in response to relatively high intracellular testosterone synthesis in cells overexpressing *AKR1C3*. Consistent with the increased synthesis of testosterone and increased DHT metabolism, a study of intraprostatic testosterone and DHT in castrated men with CRPC (obtained from transurethral resections performed to relieve obstruction) showed that levels of testosterone were not significantly reduced relative to controls with normal serum androgen levels, while DHT levels were lower than in the controls [8].

Taken together, these data indicate that testosterone synthesis from weak adrenal androgens, which occurs physiologically in the stroma and basal epithelium of normal prostate, is upregulated in CRPC cells and may provide a substantial source of ligand for AR. Consistent with this hypothesis, early studies found that about one-third of patients with CRPC had objective responses (tumor shrinkage) to adrenalectomy or hypophysectomy, and that the majority of patients improved symptomatically [71]. This surgical approach was later replaced by treatment with aminoglutethimide or ketoconazole, which both suppress adrenal androgen synthesis. However, responses are generally partial and transient, and it appears clear that even total elimination of adrenal androgen production has limited efficacy. This may reflect other alternative ligands or additional sources of androgen precursors, including the possible synthesis of androgens from cholesterol by CRPC cells [10,72], but may also reflect the eventual emergence of tumor cells that are no longer dependent on AR transcriptional activity.

A final poorly understood feature of CRPC is that it does not generally respond to treatment with AR antagonists, including high-dose (150–200 mg) bicalutamide, which can effectively block the AR when used as single agents for initial hormone therapy [31,32,73]. One explanation may be that bicalutamide (as a relatively weak competitive antagonist) can no longer effectively compete for AR binding due to increased intracellular testosterone and/or molecular changes that have markedly increased the AR affinity for agonist ligands (“hypersensitive” AR). An alternative general hypothesis is that bicalutamide can compete for AR binding in CRPC cells, but no longer functions as an antagonist. Recent studies have shown that AR can recruit corepressor proteins (NCoR and SMRT), which may contribute to the antagonist activity of bicalutamide, and that AR overexpression or removal of NCoR from the nucleus may convert bicalutamide to an agonist [17,74–77]. However, other data show that NCoR and SMRT downregulation by siRNA does not reveal bicalutamide agonist activity, and indicate that bicalutamide functions as an antagonist because it fails to mediate coactivator recruitment [78,79]. In any case, a better understanding of the molecular basis for AR antagonist resistance in CRPC is clearly important for the generation of more effective antagonists.

In summary, current data indicate that PCa cells adapt to androgen deprivation therapy by multiple mechanisms, which include increasing *AR* gene expression and androgen biosynthesis, and activation of multiple pathways that can directly or indirectly enhance AR activation by low levels of androgen. Secondary hormonal therapies with available inhibitors of androgen synthesis (ketoconazole, finasteride, dutasteride) or AR antagonists

(bicalutamide, flutamide, nilutamide) have modest efficacy, but responses may be improved with more potent agents that are in clinical trials. An alternative approach is to target AR folding and stability by the Hsp90 chaperone complex, which can be suppressed by direct Hsp90 inhibitors or indirectly by HDAC inhibitors that prevent HDAC6 mediated deacetylation of Hsp90. Finally, more data are needed to understand precisely how AR becomes sensitized to low androgen levels, and to identify agents that target these mechanisms.

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Figure 1. AR activation in normal prostate/hormone naïve PCa and reactivation after androgen deprivation therapy

Androgen binding causes a conformational change in AR that results in dissociation from Hsp90, nuclear translocation, dimerization on androgen responsive elements (AREs) in androgen regulated genes, recruitment of steroid receptor coactivator proteins (SRC1–3), and recruitment of multiple other transcriptional coactivator proteins that acetylate histone (histone acetyltransferases, HATs) (CBP/p300, pCAF), methylate histones (CARM1), unwind DNA (SWI/SNF), or recruit the RNA polymerase II complex (TRAP220). AR activity is initially suppressed by androgen deprivation therapy (ADT), but the tumor cells eventually adapt by one or more mechanisms to reactivate AR activity, resulting in (or contributing to) the emergence of castration resistant prostate cancer (CRPC).

Figure 2. Androgen synthesis and metabolism in normal prostate and prostate cancer cells

The enzymes mediating testosterone and DHT synthesis from DHEA-S, DHEA, and androstenedione from precursor steroids are expressed in normal prostate, and many are increased in CRPC (HSD3B, AKR1C3, and SRD5A1), while SRD5A2 (the type 2 5 α -reductase expressed at highest levels in normal prostate) is reduced. Enzymes mediating DHT catabolism are similarly increased, and CRPC cells may also express CYP17A1 (the enzyme inhibited by ketoconazole and abiraterone) and thereby synthesize androgens *de novo* from cholesterol.