Functional Characterization of Mutant Androgen Receptors from Androgen-independent Prostate Cancer

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INTRODUCTION

Prostate cancer is the most frequently diagnosed malignancy in men (1), and the AR plays an important role in its development (2). Most prostate cancers are androgen dependent, and treatments that decrease androgen levels (androgen ablation) are the only effective systemic therapy for this disease (3). Although the majority of tumors respond initially to androgen ablation, most recur within 2–3 years (4). Prostate cancers recurring after androgen ablation have been termed hormone refractory or AI, but secondary hormonal treatments are effective in a minority of patients (5). These secondary hormonal therapies generally either decrease the synthesis of adrenal androgens (6, 7) or block the AR directly with antiandrogens (8), indicating that AI prostate cancers are heterogeneous and that some tumors maintain a degree of hormone dependence.

The AR is expressed at normal or increased levels by most AI prostate cancers (9–12). However, the extent to which these ARs are responsible for stimulating tumor cell growth after androgen ablation is unclear. Recently, a number of ARs with mutations in the hormone binding domain have been identified in AI prostate cancers (12–15). In some cases, these mutations have been shown to alter responses to estrogen, progesterone, and/or adrenal androgens (12, 13). However, it is not yet clear whether these functional alterations account for the in vivo selection of these tumor cells.

The efficacy of androgen ablation therapy may be increased by the use of antiandrogens, which bind directly to the AR and block the effects of residual androgens (4). This type of treatment is termed complete androgen blockade, and the most commonly used antiandrogen for this purpose has been flutamide. Interestingly, up to 40% of patients who develop progressive prostate cancer after complete androgen blockade with flutamide will have a second response to discontinuation of flutamide (flutamide withdrawal response; Ref. 16). This observation indicates that flutamide directly or indirectly stimulates proliferation of prostate cancers in a subset of patients. One mechanism for direct stimulation of prostate cancers by flutamide may be through activation of mutant ARs. A prostate cancer cell line, LNCaP, with a mutant AR which is activated by flutamide, has been identified (17, 18), and the identical mutation has been reported in several patients with AI prostate cancer (14, 15).

To further assess the functional significance of AR mutations that have been isolated from patients with AI prostate cancer, this study systematically investigated the responses of these ARs to steroid hormones and antiandrogens. The results indicate that these mutations do not enhance AR responses to low concentrations of DHT, the most active androgen in normal
prostate. Responses to androstenedione, a major adrenally derived androgen, were similarly not increased by any of the mutations. In contrast, mutant ARs strongly activated by flutamide were identified. These results indicate that AR mutations may be selected for by flutamide in vivo and that this process may contribute to the flutamide withdrawal response. Treatment strategies that could minimize selection for these mutations are discussed.

MATERIALS AND METHODS

AR Expression Plasmids. An SV40-based expression vector containing the wild-type human AR, pARO, was kindly provided by Dr. Albert Brinkmann (Erasmus University, Rotterdam, the Netherlands; Ref. 19). Restriction sites in the AR were used to replace fragments of the wild-type AR with the corresponding fragments containing single-point mutations. The transferred fragments containing the point mutations were sequenced completely to exclude any second-site mutations. The isolation and cloning of a series of mutant ARs derived from patient samples were described previously (12). These included mutations in codon 902 (Gln to Arg, Q902R), codon 877 (Thr to Ser, T877S), codon 874 (His to Tyr, H874Y), and codon 721 (Ala to Thr, A721T).

Additional previously described mutations analyzed in this study were in codons 877 (Thr to Ala, T877A) and 715 (Val to Met, V715M). The T877A mutant AR is expressed by the LNCaP line (18) and was isolated by PCR amplification from this cell line. The V715M mutation was isolated independently from a patient with AI prostate cancer and was identical to the V715 mutation described previously (13). The luciferase reporter plasmid used in these studies, MMtvA3LUC, derived from pHHLUC (20), was regulated by the androgen-responsive promoter plasmid used in these studies, MMTVpA3LUC, derived from a patient with AI prostate cancer and was identical to the V715 mutation described previously (13). The luciferase reporter plasmid used in these studies, MMtvA3LUC, derived from pHHLUC (20), was regulated by the androgen-responsive elements in the mouse mammary tumor virus long terminal repeat (kindly provided by Dr. Richard Pestell, Northwestern University Medical School, Chicago, IL).

Transfections. CV-1 cells grown in DMEM with 5% FCS in 24-well plates were cotransfected with the AR, MMtvA3LUC, and pSv-β-galactosidase (Promega Corp., Madison, WI) plasmids using calcium phosphate (Mammalian Cell Transfection kit; Specialty Media, Lavalette, NJ), as described by the manufacturer. Each well of CV-1 cells contained 460 μl of DMEM with 5% FCS and received 40 μl of a 1-m1 mixture containing 5 μg of AR, 10 μg of LUC, and 5 μg of β-galactosidase. Approximately 16 h after transfection, cells were washed, and 450 μl of DMEM containing 5% charcoal-stripped (steroid hormone-free) FCS (Hyclone Laboratories, Logan, UT) were added per well for 4 h. The specific hormones were then added in 50 μl, and the cells were incubated for another 24 h. The cells were then lysed, and luciferase and β-galactosidase activities were measured. Controls included untransfected cells and transfected cells without the addition of hormone.

Steroid hormones tested included DHT, androstenedione, progesterone, and estradiol (Steraloid, Inc., Wilton, NH). Androgens tested included hydroxyflutamide (the active metabolite of flutamide, a gift from Shering-Plough, Kenilworth, NJ), nilutamide (Roussell-Uclaf Company, Romainville, France), and bicalutamide (ICI 176,334, Casodex; Zeneca Pharmaceuticals). Background levels were determined in extracts of mock-transfected cells, and luciferase activity was divided by 105 to yield RLUs. All determinations were done in quadruplicate in individual experiments, and results of representative experiments are shown.

RESULTS

Stimulation of Wild-Type versus Mutant ARs by Androgens. CV-1 cells were cotransfected with plasmids expressing wild-type or mutant ARs and a luciferase reporter plasmid, and luciferase activity was then determined after incubation with varying concentrations of DHT. Minimal luciferase activity was observed for the wild-type AR or any mutant AR in the absence of hormone (Fig. 1). In a series of transfection experiments, the wild-type AR responded to DHT at less than 0.1 nM DHT, and maximal responses were noted between 1 and 10 nM DHT (Fig. 1). A decrease in activity was observed at high DHT concentrations (100 nM), but this is unlikely to be of physiological significance (21).

Each mutant AR tested in these experiments, except the Q902R mutant, had a significant response to DHT (Fig. 1). However, there was no increase in the response to DHT by any of the mutant ARs relative to the wild-type AR, except at the highest DHT concentration (100 nM). Moreover, the DHT concentration required for half-maximal activity was <0.01 nM for the wild-type AR and at least one log greater for the mutant ARs (Fig. 1). Therefore, these results indicate that tumor cells expressing these mutant ARs were not selected based upon increased responses to low levels of DHT.

The adrenal gland may be an important source of androgens in prostate cancer following androgen ablation therapy (22), and the V715M mutant AR was reported previously to have an increased response to androstenedione (13), a major adrenal androgen. Therefore, the responses of mutant ARs to androstenedione were assessed. At high androstenedione concentrations (100 nM), the activity of the wild-type AR was greater than that of the mutant ARs (Fig. 2). At low androstenedione concentrations, the activities of the V715M, H874Y, and T877S mutants were similar to, but not significantly greater than, the wild-type AR. The activities of the T877A, Q902R, and A721T ARs were less than the wild-type AR at all androstenedione concentrations examined (Fig. 2 and data not shown).

Responses to Estradiol and Progesterone by Mutant ARs. Responses by several mutant ARs to estradiol and/or progesterone were reported previously (12, 13, 18, 23). The T877A, T877S, and H874Y mutants were shown to have increased responses to both estradiol and progesterone relative to the wild-type AR (12, 18, 23), whereas the V715M mutant was shown to have an increased response to progesterone (13). The A721T and Q902R ARs have not been studied previously and were examined here. Fig. 3 shows that neither of these ARs demonstrated enhanced activation by progesterone or estradiol relative to the wild-type AR.
Fig. 1 Stimulation of wild-type compared to mutant ARs by DHT. Wild-type (WT) or mutant ARs were transiently expressed in CV-1 cells with a luciferase reporter gene and incubated in the presence of various concentrations of DHT. The luciferase activity expressed in RLU's was normalized for β-galactosidase activity and divided by 10³ to yield a standardized RLU. All determinations were done in quadruplicate, and the mean results of a representative experiment are shown. SEs were all below 25% of the mean, except for V715M at 0.01 nM, which was 29%.

Fig. 2 Stimulation of wild-type and mutant ARs by androstenedione. Wild-type (WT) or mutant ARs were transiently expressed in CV-1 cells with a luciferase reporter gene and incubated in the absence or presence of various concentrations of androstenedione. All determinations were done in quadruplicate, and the mean results of a representative experiment are shown. SEs were below 20%, except Q902R at 100 nM and T877S at 0.1 nM, which were 22, 27, and 27%, respectively.

Responses to Antiandrogens. A modest but reproducible response by the wild-type AR was observed at high concentrations of hydroxyflutamide (Fig. 4A). Similar weak responses to nilutamide and hydroxyflutamide were observed with the A721T AR (Fig. 4A). Hydroxyflutamide stimulated a more significant increase in the activity of the V715M AR, although this response was still approximately 20-fold less than the maximal response that could be induced by DHT from the wild-type AR. Moreover, it was only observed at the highest hydroxyflutamide concentration.

Stimulation of the H874Y and T877S ARs by hydroxyflutamide was more substantial (Fig. 4B). Activation of these ARs was clearly observed at 100 nM hydroxyflutamide, and the maximal response was only about 4-fold lower than the response to DHT. The H874Y and T877S ARs could also be activated by nilutamide. In contrast, neither mutant nor wild-type ARs were activated by bicalutamide (Fig. 4B).

The ability of bicalutamide to inhibit the T877S and H874Y mutant ARs was next determined. Fig. 5 shows that bicalutamide remains capable of antagonizing the response to androgens (DHT and androstenedione), as well as to progesterone and estradiol, by the mutant ARs.

DISCUSSION
This study addressed whether enhanced responses to certain hormones or drugs might play a role in vivo in stimulating the growth of AI prostate cancers expressing particular mutant
Fig. 3 Responses of mutant ARs to estradiol and progesterone. Wild-type (WT) or mutant ARs (Q902R or A721T) were transiently expressed in CV-1 cells with a luciferase reporter gene and incubated in the absence or presence of estradiol (E) or progesterone (P) at the concentrations indicated. All determinations were done in quadruplicate, and the means of a representative experiment are shown: bars, SE. The maximal response to 0.1 μM DHT for the WT AR in this experiment was 341 RLU.

ARs. In the absence of hormone, there was no detectable activation observed, indicating that none of the ARs are constitutively active. Although each of the mutant ARs, except Q902R, responded to DHT, an enhanced response to this hormone relative to the wild-type AR was not observed. Most significantly, the response of the wild-type AR was markedly greater than the mutant ARs at low DHT concentrations. Therefore, whereas the mutant ARs may respond to DHT, these results do not support the hypothesis that tumor cells expressing these mutant ARs would have an increased response in vivo to the low levels of DHT present after androgen ablation.

Responses to androstenedione were similarly observed with each of the ARs, including a weak response by Q902R. In contrast to the DHT responses, several of the mutant ARs were comparable to the wild-type AR at androstenedione concentrations in the 1 nM range. Although small differences in responses to androstenedione could be of significance in vivo, these experiments suggest that large increases in androstenedione responses do not account for the selection of these mutations. It should be noted that a previous report indicated that the response of the V715M AR to androstenedione was approximately 4-fold greater than the wild-type at concentrations of 10 and 50 nM (13). However, a subsequent report from the same authors showed no significant difference (24) and is consistent with the results reported here.

Three of the mutant ARs (V715M, H874Y, and T877S) were activated by hydroxyflutamide and nilutamide. In contrast, these mutations did not alter the response to bicalutamide, which remained an effective antiandrogen. The mutant AR from the LNCaP cell line (T877A) was similarly activated by hydroxyflutamide and inhibited by bicalutamide (17, 18, 25). Activation of the V715M AR by hydroxyflutamide was not observed in a previous study (13), but a more recent study from the same authors concluded that this AR could be stimulated by high concentrations of hydroxyflutamide (24). Finally, it should be noted that the ARs that were strongly stimulated by hydroxyflutamide, T877S and H874Y, were derived from patients who were treated previously with flutamide (12). The V715M AR was also identified in a patient who had received flutamide (13).

It is clear that the functional assay system used here has its limitations and may not fully predict the behavior of these mutant ARs in vivo in prostate cells for a number of reasons. AR mutations could alter interactions with certain AR-associated coactivator proteins that occur specifically in prostate cells. The
AR may have additional functions in prostate cells, which are not reflected in these experiments. The intracellular concentrations of DHT and other hormones in prostate cancer cells are also unknown. Nonetheless, this assay system does indicate that several of the AR mutations alter the AR in a way that could result in positive selection for the corresponding tumor cells in the setting of flutamide treatment. This suggests that AR mutations of this type could contribute to flutamide failures and the flutamide withdrawal syndrome.

These experiments further suggest that flutamide may function as a weak agonist for wild-type ARs (Fig. 4A). The weak agonist activity of hydroxyflutamide was also noted recently by another group (26). This agonist activity may not be significant for primary androgen-dependent prostate cancers, which develop in the setting of relatively high DHT levels. However, androgen ablation could possibly select for cells with wild-type ARs in which this activity is significant due to AR overexpression (11, 27), changes in AR-associated proteins, or other mechanisms. The development of such a population of flutamide-dependent cells expressing wild-type ARs may be another contributor to the flutamide withdrawal response. Moreover, cells that are initially flutamide dependent could progress to varying degrees of flutamide independence and also contribute to flutamide failure (28). Finally, it should be noted that these agonist effects on wild-type ARs, as well as the agonist effects on mutant ARs, occur at levels well within the serum concentrations of hydroxyflutamide observed in patients (29).

The observations that bicalutamide remains an antagonist for each of the flutamide-reactive mutant ARs identified here and has no weak agonist activity on wild-type ARs suggest that this drug may be useful in some patients with AI prostate cancer. Moreover, if flutamide treatment alters the biology of subsequent AI prostate cancers and selects for hormone-dependent tumor cells, then bicalutamide may be particularly effective in flutamide-treated patients. However, the fraction of patients with AI prostate cancer treated initially with androgen ablation or androgen ablation plus flutamide who harbor functionally significant AR mutations remains unclear (12–15). The proportion of flutamide-treated patients who respond to flutamide withdrawal, a possible indicator of AR alterations, has also varied widely in a series of studies (30). Finally, recent reports of bicalutamide withdrawal responses (31) suggest that this drug may similarly select for its own particular spectrum of AR alterations after long-term use. Therefore, whether bicalutamide can be useful in any significant fraction of patients with AI prostate cancer will need to be determined in clinical trials.

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REFERENCES


Fig. 5 Inhibition of mutant ARs by bicalutamide. Mutant ARs were transiently expressed in CV-1 cells with a luciferase reporter gene and incubated in the presence of DHT (1 nM), androstenedione (A; 10 nM), estradiol (E; 10 nM), or progesterone (P; 10 nM), with (+) or without (−) the addition of bicalutamide at a concentration of 100 nM. All determinations were done in quadruplicate, and the means from a representative experiment are shown; bars, SE.
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