**The Androgen Axis in Recurrent Prostate Cancer**

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Abstract

**Purpose.** Prostate cancer that recurs during androgen deprivation therapy is referred to as androgen-independent. High levels of expression of androgen receptor and androgen receptor-regulated genes in recurrent prostate cancer suggest a role for androgen receptor and its ligands in prostate cancer recurrence.

**Experimental Design.** Recurrent prostate cancer specimens from 22 men whose prostate cancer recurred locally during androgen deprivation therapy and benign prostate specimens from 48 men who had received no prior treatment were studied. Androgen receptor expression was measured using monoclonal antibody and automated digital video image analysis. Tissue androgens were measured using radioimmunoassay.

**Results.** Epithelial nuclei androgen receptor immunostaining in recurrent prostate cancer (mean optical density, 0.284 ± SD 0.115 and percentage positive nuclei, 83.7 ± 11.6) was similar to benign prostate (mean optical density, 0.315 ± 0.044 and percentage positive nuclei, 77.3 ± 13.0). Tissue levels of testosterone were similar in recurrent prostate cancer (2.78 ± 2.34 pmol/g tissue) and benign prostate (3.26 ± 2.66 pmol/g tissue). Tissue levels of dihydrotestosterone, dehydroepiandrosterone, and androstenedione were lower (Wilcoxon, P = 0.0000068, 0.00093, and 0.0089, respectively) in recurrent prostate cancer than in benign prostate, and mean dihydrotestosterone levels, although reduced, remained 1.45 nm. Androgen receptor activation in recurrent prostate cancer was suggested by the androgen-regulated gene product, prostate-specific antigen, at 8.80 ± 10.80 nmol/g tissue.

**Conclusions.** Testosterone and dihydrotestosterone occur in recurrent prostate cancer tissue at levels sufficient to activate androgen receptor. Novel therapies for recurrent prostate cancer should target androgen receptor directly and prevent the formation of androgens within prostate cancer tissue.

Introduction

In the United States in 2003, an estimated 220,900 new cases of prostate cancer were diagnosed and 28,900 men died from prostate cancer (1). Despite earlier detection (2), ~30% of men treated with curative intent will suffer tumor recurrence. These men as well as those who present with locally advanced or metastatic prostate cancer can be palliated by androgen deprivation therapy, a treatment that remains unimproved since its discovery >50 years ago (3). Regardless of the initial responsiveness to androgen deprivation therapy, almost all patients succumb to recurrent prostate cancer because it responds poorly to all of the known therapies.

The androgen receptor may play a central role in the development and progression of recurrent prostate cancer (4–6). Although variation of expression of androgen receptor protein has been correlated with response to androgen deprivation therapy (7–10), androgen receptor expression appears similar in androgen-dependent and recurrent prostate cancer (11, 12). On a molecular level, mutations have been reported in androgen-dependent prostate cancer with a frequency ranging from 0 (13) to 44% (14) and in recurrent prostate cancer with a frequency ranging from 0 (15) to 50% (16). When characterized functionally, most of the mutant androgen receptors retain transcriptional activity in response to androgens and some have altered steroid-binding specificity that changes the spectrum of ligands capable of activating androgen receptor (16–20).

We examined 22 specimens of recurrent prostate cancer sufficient for measurement of androgen receptor protein expression in all and tissue androgens in 15 specimens. Levels of androgen receptor expression and tissue androgens were compared with levels of androgen receptor expression and tissue androgens in benign prostate of untreated patients. We report that androgen receptor protein is expressed at similar levels in recurrent prostate cancer and androgen-stimulated benign prostate. Many have assumed that androgen receptor is stabilized by a ligand-independent mechanism because testicular androgens are unavailable after medical or surgical castration. We tested the alternative hypothesis and measured tissue levels of dihydrotestosterone (DHT), the preferred ligand, and testosterone and the adrenal androgens, dehydroepiandrosterone (DHEA), DHEA-sulfate, and androstenedione (ASD). We report that tis-
malignant nuclei were segmented automatically, classified as immunopositive or immunonegative, and immunostaining intensity measured using color image analysis software. Nineteen specimens of recurrent prostate cancer were compared with 16 specimens of benign prostate acquired by transurethral resection and processed routinely, because androgen receptor immunostained less intensely in benign prostate acquired from radical prostatectomy specimens due to differences in fixation (21). Differences in mean optical density (MOD) and percentage of positive nuclei between recurrent prostate cancer and benign prostate were evaluated using 2-sided Wilcoxon analysis. Linear regression analysis was used to search for correlation between the androgen receptor expression parameters, androgen receptor MOD and percentage of positive nuclei, and the clinical parameters, age, prostate-specific antigen (PSA), and Gleason sum at the time of tissue procurement and survival from the time androgen deprivation therapy was begun.

**Measurement of Tissue Androgens.** Frozen specimens of recurrent prostate cancer and benign prostate were assayed for total levels of testosterone, DHT, ASD, DHEA, dehydroepiandrosterone sulfate (DHEA-SO₄), sex hormone binding globulin (SHBG), and PSA (Diagnostic Systems Laboratories, Inc., Webster, TX; Diagnostic Products Corporation, Los Angeles, CA). DHT was extracted into 98% hexane, 2% ethanol, centrifuged, evaporated, and reconstituted in assay buffer after [³H]DHT was added as an internal standard to correct for recovery. The procedure reduced testosterone cross-reactivity to 0.02%, and recovery was 70%. DHT values were reported after correction for recovery. The detection limits in pmol/g tissue were testosterone 0.87, DHT 0.14, ASD 0.52, DHEA 0.70, PSA 0.0080, SHBG 0.10, and DHEA-SO₄ 34 in recurrent prostate cancer and 17 in benign prostate. The assays were highly specific for their respective analytes with the exception of the DHEA-SO₄ assay, which had 41% cross-reactivity with DHEA. When analyte levels were below the limit of detection, the limit of detection (not zero) was used for data description and statistical testing that may have introduced bias in favor of not finding a difference. For comparison of DHEA-SO₄ values between recurrent prostate cancer and benign prostate, a lower limit of detection (zero) was used for data description and statistical testing that may have introduced bias in favor of not finding a difference. For comparison of DHEA-SO₄ values between recurrent prostate cancer and benign prostate, a lower limit of detection of 34 pmol/g tissue was used to prevent bias. Two-sided Wilcoxon analysis was used to compare tissue levels of analytes between recurrent prostate cancer and benign prostate, and, in recurrent prostate cancer, between patients who did and did not receive antiandrogens. Linear regression analysis was used to search for correlation between tissue levels of analytes and clinical parameters, survival, androgen receptor MOD, and percentage of positive nuclei.

**Results.**

**Patient Clinical Characteristics.** Twenty two patients 57–86 years of age (mean, 73 ± 8 years) demonstrated clinical evidence of recurrent prostate cancer (Table 1). All suffered urinary retention from local recurrence that occurred from 7 to 92 months (mean, 37 ± 24 months) after medical (10 patients) or surgical (11 patients) androgen deprivation therapy. All but patient 15 had increasing serum levels of PSA and all of the men had castrate levels of serum testoster-

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7 Unpublished observations.
Androgen Receptor Expression. Immunohistochemistry (Fig. 1) revealed androgen receptor immunostaining in 19 samples of recurrent prostate cancer (MOD, 0.284 ± 0.115 and percentage of positive nuclei, 83.7 ± 11.6) that was similar to that of 16 samples of benign prostate (MOD, 0.315 ± 0.044 and percentage of positive nuclei, 77.3 ± 13.0; \( P = 0.48 \) for MOD and 0.25 for percentage of positive nuclei; Fig. 2; Table 1). Among patients with recurrent prostate cancer, there was no significant relationship between MOD or percentage of positive nuclei of androgen receptor immunostaining and age, serum PSA, Gleason sum, or survival. Neither androgen receptor MOD nor percentage of positive nuclei differed between 4 patients who were treated with flutamide and 18 patients who were not. Among patients with benign prostate, there was no significant relationship between androgen receptor MOD or percentage of positive nuclei and age.

Tissue Androgens. Tissue levels of testosterone were similar in recurrent prostate cancer (mean, 2.78 nm) and benign prostate (mean, 3.26 nm; \( P = 0.21 \)). Tissue levels of DHT, DHEA, DHEA-SO₄, and ASD were lower in recurrent prostate cancer than in benign prostate (Wilcoxon, 2-sided \( P = 0.0000068, 0.00093, 0.0608, \) and 0.0089, respectively; Tables 1 and 2; Fig. 3), although tissue levels of DHT averaged 1.45 nm in recurrent prostate cancer and 8.13 nm in benign prostate. SHBG levels were similar in recurrent prostate cancer and benign prostate (\( P = 0.65 \)). Tissue levels of PSA in recurrent prostate cancer were \( \sim 1/10 \) the level measured in benign prostate (\( P = 0.00000057 \)).

Among patients with recurrent prostate cancer, tissue levels of androgens, SHBG, and PSA were unrelated to the clinical descriptors age, serum PSA, Gleason sum, and survival and the androgen receptor expression descriptors MOD and percentage of positive nuclei. Recurrent prostate cancer tissue levels of androgens, SHBG, and PSA did not differ between 3 patients who received flutamide and 12 patients who did not. In particular, tissue levels of DHT were similar (\( P = 0.29 \)) in the two groups (flutamide, 3.75 ± 3.58 pmol/g tissue, range 0.40–7.53 pmol/g tissue; no flutamide, 0.87 ± 0.53 pmol/g tissue, range 0.37–2.17 pmol/g tissue).

Among patients with benign prostate, 21% of the variation in ASD was explained by age (\( r = 0.46; P = 0.0083 \)). Age was not related to tissue levels of protein, SHBG, or other androgens. Tissue levels of androgens SHBG and PSA were unrelated to the androgen receptor expression descriptors MOD and percentage of positive nuclei.

### Table 1. Androgen receptor expression and tissue androgens in recurrent prostate cancer

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<th>Gleason sum</th>
<th>Bone metastases</th>
<th>Androgen deprivation therapy</th>
<th>Interval (mo) from androgen deprivation therapy to tissue acquisition</th>
<th>Survival (mo) after tissue acquisition</th>
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* PSA, prostate-specific antigen; 0, absent; 1, present; 1° hypogonad, primary hypogonadism; AA, African American; ASD, androstenedione; BLD, below limit of detection; CA, Caucasian American; DES, diethylstilbestrol; DHEA, dehydroepiandrosterone; DHEA-SO₄, dehydroepiandrosterone sulfate; DHT, dihydrotestosterone; flu, flutamide; LHRH, luteinizing hormone-releasing hormone; mo, months; ND, not done, insufficient tissue; orch, orchietomy; SHBG, sex hormone binding globulin; T, testosterone; X = unknown; yrs, years.
Discussion

The hallmark of recurrent prostate cancer is clinical progression despite surgical or medical castration and antiandrogen therapy. Despite the ineffectiveness of androgen deprivation therapy, evidence supports a role for androgen receptor in recurrent prostate cancer. Androgen receptor is expressed in recurrent prostate cancer (11, 12), androgen receptor immunochemistry and image analysis, activating levels of tissue androgen receptor protein. Van der Kwast et al. (11) reported that primary and recurrent tumors did not appear different by androgen receptor immunohistochemistry using antigen-retrieval, monoclonal androgen receptor antibody, and visual assessment of immunostaining intensity. High levels of expression of androgen receptor mRNA have also been reported using quantitative reverse transcription-PCR when radical prostatectomy specimens were studied after various intervals of complete androgen blockade (32).

Androgen receptor protein levels increase when stabilized by ligand, and the preferred ligand for androgen receptor is DHT. The persistence of androgen receptor protein in prostate cancer independent of circulating androgen levels is surprising and has led to the suggestion that androgen receptor is activated and, hence, stabilized by ligand-independent mechanisms (4–6). Data presented herein indicate that, despite removal of testicular androgens, the level of testosterone in recurrent prostate cancer tissue is unchanged relative to levels in benign tissue. Furthermore, although the mean DHT level was decreased to 18% of the level in benign prostate tissue, patient tissue DHT levels in most patients were well above levels required to activate androgen receptor based on studies in prostate cancer cell lines (33, 34). Forti et al. (35) reported 90% decrease in tissue levels of DHT to 0.48 ng/g tissue (equivalent to 1.66 nM) in benign prostate after 3 months of luteinizing hormone-releasing hormone (LHRH) treatment. They cautioned, “long-term treatment (of men with prostate cancer) with GnRH agonists . . . may not reduce intraprostatic androgen concentrations to undetectable levels.” Results obtained using radioimmunoassay were confirmed in a subset of 5 benign prostate and 5 recurrent prostate cancers assayed commercially using ELISA (ProteEx, Inc., Woodlands, TX; data not shown). Others have reported that testosterone and DHT levels were similar in benign prostate when measured using radioimmunoassay and two different mass

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<th>% positive nuclei</th>
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<th>PSA (pmol/g tissue)</th>
<th>SHBG (pmol/g tissue)</th>
<th>T (pmol/g tissue)</th>
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Table 1 Continued

Continued
spectrometry methods (36). The persistence of activating levels of testosterone and DHT in recurrent prostate cancer is surprising and additionally supports a role for androgen receptor in prostate cancer progression.

It is not clear to what extent the testosterone and DHT in recurrent prostate cancer tissue derives from adrenal androgens or other steroid precursors. Harper et al. (37) reported that small amounts of DHT were formed from DHEA and DHEA-SO_4 in benign prostate. Belanger et al. (38) suggested that persistent levels of prostatic DHT after castration (39–41) are derived from adrenal DHEA, DHEA-SO_4, and ASD in the prostate. Adult men have circulating DHEA levels of ~25 nm derived predominantly from the adrenal glands (42). Serum DHEA-SO_4 can be 300–500 times this concentration, and a sulfatase is present in human prostate that converts DHEA-SO_4 to DHEA (43), which may serve as a precursor to testosterone (37). We found mean tissue levels of DHEA and DHEA-SO_4 of 63 and 81 nm, respectively, in benign prostate that are similar to a prior report (43). Recurrent prostate cancer tissue levels of ASD and DHEA were ~50% the levels in benign prostate that suggest sufficient substrate is available if the appropriate steroid metabolizing enzymes are present. The increased ratio of testosterone: DHT in recurrent prostate cancer compared with benign prostate tissue suggests that 5α-reductase activity may be altered in recurrent prostate cancer.

Tissue androgen levels in recurrent prostate cancer, although not reported previously, appear to have a complex relationship to castration and antiandrogen therapy. Prostate cancer tissue DHT levels decreased from 5.24 ng/g tissue in noncastrated men 55–68 years of age to 2.7 ng/g tissue in men who were castrated 2–12 months before radical prostatectomy (38). Among castrated men receiving flutamide 250 mg three times daily for 2 months before prostatectomy, tissue DHT was undetectable. It was postulated that flutamide, by competing for high affinity DHT binding to androgen receptor, decreased prostate DHT levels by increasing its degradation (39). In contrast, we found that tissue levels of all of the androgens except testosterone were reduced in recurrent prostate cancer tissue after castration. Furthermore, tissue androgen levels were similar between patients who received flutamide and those who did not. Thus, in recurrent prostate cancer, testosterone and DHT were detectable whether medical or surgical castration was used alone or combined with flutamide. Our findings are consistent with current clinical experience. A meta-analysis of clinical trials comparing LHRH agonists and antiandrogens versus LHRH agonists alone (44), and a study comparing orchietomy

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**Fig. 1** Photomicrographs of androgen receptor expression. Androgen receptor expression was similar visually in benign prostate (left) and recurrent prostate cancer (right) obtained by transurethral resection, fixed in formalin, embedded in paraffin, antigen-retrieved, and immunostained with antiandrogen receptor monoclonal antibody. Photomicrographs were reduced from ×400.

**Fig. 2** Quantitative analysis of androgen receptor expression. Androgen receptor expression in benign prostate (□) and recurrent prostate cancer (■) was measured using quantitative digital video image analysis. Mean absorbance (mean optical density) and percentage of positive nuclei (% nuclei) for specimens of recurrent prostate cancer from 19 men and specimens of benign prostate from 16 men were similar statistically and described by means; bars, ±SDs.
and antiandrogens versus orchiectomy alone (45) demonstrated no survival benefit to combination therapy.

DHT is considered the active androgen in the prostate (46–50), and the DHT in prostate tissue after castration is likely to be androgenic (51). Studies on prostate cancer cell lines indicated that mean levels of 2.78 nm testosterone and 1.45 nm DHT measured in recurrent prostate cancer tissue should be sufficient to activate androgen receptor (34). Recurrent prostate cancer cell lines CWR-R1 and LNCaP-C4-2 have an increased sensitivity to the growth promoting effects of DHT, which is 4 orders of magnitude lower than the DHT concentrations measured in recurrent prostate cancer tissue (assuming 1 mg DNA/g tissue) in aspirated benign prostate tissue (3198 pmol/g tissue). Yang et al. (60) reported tissue PSA levels in androgen-stimulated and recurrent prostate cancer tissue were only 9% of levels in benign tissue, but androgen receptor appeared activated based on the similar interaction between the NH₂-terminal domain of androgen receptor and SRC1 (55). Growth factor kinase signaling pathways may activate androgen receptor directly (56, 57) or indirectly by regulating coactivator interactions with androgen receptor (58).

Although clinical specimens of recurrent prostate cancer demonstrated high levels of androgen receptor protein expression, data from in vitro assays are cited to suggest that the tissue androgen levels measured in recurrent prostate cancer are sufficient for androgen receptor activation. PSA levels in recurrent prostate cancer tissue were only 9% of levels in benign tissue, but androgen receptor appeared activated based on the similar PSA levels in androgen-stimulated and recurrent prostate cancer. Stege et al. (59) reported a mean PSA level of 4973 pmol/g tissue (assuming 1 mg DNA/g tissue) in aspirated benign prostate tissue, which was similar to the level we measured for recurrent prostate cancer (297 pmol/g tissue). Yang et al. (60) reported tissue PSA levels in recurrent prostate cancer tissue. Yang et al. (60) reported tissue PSA levels in transurethral resection specimens of 1952 pmol/g tissue. Yang et al. (60) reported tissue PSA levels in recurrent prostate cancer tissue were only 9% of levels in benign tissue, but androgen receptor appeared activated based on the similar interaction between the NH₂-terminal domain of androgen receptor and SRC1 (55). Growth factor kinase signaling pathways may activate androgen receptor directly (56, 57) or indirectly by regulating coactivator interactions with androgen receptor (58).
Recurrent Prostate Cancer Tissue Androgen Levels

tate and mean dihydrotestosterone levels, although reduced, remained testosterone were similar in recurrent prostate cancer and benign pros-
dihydrotestosterone, dehydroepiandrosterone, and androstenedione were lower (Wilcoxon, **p** < 0.00000068, 0.00093, and 0.0089, respectively) in recurrent prostate cancer than in benign prostate. Tissue levels of testosterone were similar in recurrent prostate cancer and benign pros-
and mean dihydrotestosterone levels, although reduced, remained 1.45 nM.

Fig. 3 Tissue androgen levels. Tissue levels of sex hormone binding globulin (SHBG), testosterone (T), dihydrotestosterone (DHT), androstenedione (ASD), dehydroepiandrosterone (DHEA), and dehydroepiandrosterone sulfate (DHEA-SO₄) in recurrent prostate cancer (□) and benign prostate (■). The measures of each analyte for specimens of recurrent prostate cancer from 16 men and specimens of benign prostate from 32 men were described by means; bars, ±SD. Tissue levels of dihydrotestosterone, dehydroepiandrosterone, and androstenedione were lower (Wilcoxon, **p** = 0.00000068, 0.00093, and 0.0089, respectively) in recurrent prostate cancer than in benign prostate. Tissue levels of testosterone were similar in recurrent prostate cancer and benign pros-

presence of PSA in recurrent prostate cancer is consistent with the presence of an activated androgen receptor. In addition, PSA, as well as other androgen-regulated genes, were expressed before and after castration in tumor models of androgen-dependent prostate cancer as shown by differential expression (26), subtractive hybridization (28), and cDNA microarray (29).

Finally, SHBG is produced by prostate cells (61) and binds a membrane receptor in prostate (62). Upon binding hormone, SHBG was reported to initiate an intracellular signal that increased cyclic AMP levels and modulated androgen action in the prostate (63, 64). We found that SHBG tissue levels were similar in benign prostate and recurrent prostate cancer.

Studies presented herein confirm quantitatively that androgen receptor protein levels are similar in androgen-stimulated benign prostate and recurrent prostate cancer. In recurrent prostate cancer, the high levels of androgen receptor protein may result from stabilization by tissue androgens. PSA expression suggests that androgen receptor is not only stabilized but activated by tissue androgens in the absence of circulating androgens. Taken together, these findings suggest prostate cancer that recurs during androgen deprivation therapy is not “androgen-independent” but continues to depend on androgen for growth. The substrates and metabolic pathways (65) responsible for maintenance of functional tissue levels of testosterone and DHT in recurrent prostate cancer remain to be clarified. These findings in recurrent CaP from the primary site may not apply to CaP metastases where androgen metabolism is independent of the prostatic microenvironment. Therapies that target androgen receptor directly using androgen receptor ribozymes or antiandrogen receptor antibodies inhibited growth of both androgen-sensitive and recurrent prostate cancer in vitro (66). New ther-

apisies that target androgen receptor directly and prevent formation of androgens within prostate cancer tissue may offer the most effective approach to prolong remission of recurrent prostate cancer.

References


The Androgen Axis in Recurrent Prostate Cancer

James L. Mohler, Christopher W. Gregory, O. Harris Ford III, et al.


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