Intraprostatic Androgens and Androgen-Regulated Gene Expression Persist after Testosterone Suppression: Therapeutic Implications for Castration-Resistant Prostate Cancer

Elahe A. Mostaghel,1,2 Stephanie T. Page,2,5 Daniel W. Lin,3,5 Ladan Fazli,6 Isa M. Coleman,1 Lawrence D. True,4 Beatrice Knudsen,5 David L. Hess,7 Colleen C. Nelson,6 Alvin M. Matsumoto,6,5 William J. Bremner,1 Martin E. Gleave,1 and Peter S. Nelson1

Abstract

Androgen deprivation therapy (ADT) remains the primary treatment for advanced prostate cancer. The efficacy of ADT has not been rigorously evaluated by demonstrating suppression of prostatic androgen activity at the target tissue and molecular level. We determined the efficacy and consistency of medical castration in suppressing prostatic androgen levels and androgen-regulated gene expression. Androgen levels and androgen-regulated gene expression (by microarray profiling, quantitative reverse transcription-PCR, and immunohistochemistry) were measured in prostate samples from a clinical trial of short-term castration (1 month) using the gonadotropin-releasing hormone antagonist, Acyline, versus placebo in healthy men. To assess the effects of long-term ADT, gene expression measurements were evaluated at baseline and after 3, 6, and 9 months of neoadjuvant ADT in prostatectomy samples from men with localized prostate cancer. Medical castration reduced tissue androgens by 75% and reduced the expression of several androgen-regulated genes (NDRG1, FKBP5, and TMPRSS2). However, many androgen-responsive genes, including the androgen receptor (AR) and prostate-specific antigen (PSA), were not suppressed after short-term castration or after 9 months of neoadjuvant ADT. Significant heterogeneity in PSA and AR protein expression was observed in prostate cancer samples at each time point of ADT. Medical castration based on serum testosterone levels cannot be equated with androgen ablation in the prostate microenvironment. Standard androgen deprivation does not consistently suppress androgen-dependent gene expression. Suboptimal suppression of tumoral androgen activity may lead to adaptive cellular changes allowing prostate cancer cell survival in a low androgen environment. Optimal clinical efficacy will require testing of novel approaches targeting complete suppression of systemic and intracrine contributions to the prostatic androgen microenvironment.

Introduction

Androgens are important mediators of transcriptional pathways controlling the proliferation, differentiation, and apoptosis of normal and neoplastic prostate cells and play a critical role in the development and progression of prostate cancer. Androgen deprivation therapy (ADT) is considered standard systemic treatment for advanced prostate cancer, and is being increasingly used in neoadjuvant and adjuvant protocols for the treatment of high-risk, early-stage disease. Remarkably, the efficacy of ADT has not been rigorously evaluated in vivo by demonstrating effective suppression at the target tissue and molecular level, either by establishing complete ablation of tissue androgen levels or abrogation of androgen-mediated gene expression. Furthermore, heterogeneity in the clinical response to initial ADT and secondary hormonal manipulations suggests that patient- and/or tumor-specific factors modulate the degree of androgen deprivation achieved within the tumor and surrounding microenvironment.

Although the adequacy of ADT is routinely assessed based on achieving castrate levels of serum testosterone, accumulating evidence suggests that despite medical castration, residual androgens are present within the prostate at levels capable of activating the androgen receptor (AR) and supporting continued expression of androgen-dependent genes (1–5). A study of patients with benign prostatic hypertrophy (BPH) found that prostatic testosterone and dihydrotestosterone levels were decreased by only 75% to 80% after achieving castrate serum testosterone levels with gonadotropin-releasing hormone agonist therapy (2). More recently, Mohler et al. (3) reported that medically or surgically castrate patients with recurrent prostate cancer actually had prostatic testosterone levels equivalent to those of patients with BPH, whereas intraprostatic dihydrotestosterone levels were only reduced by ~75%. Importantly, in vitro and in vivo studies have shown that dihydrotestosterone levels in the range observed in the prostatic tissue of castrated patients (0.5–1.0 ng/g) are sufficient to activate the AR and stimulate expression of androgen-regulated genes (3, 6–9).

To date, human studies examining the association between prostate gene expression and tissue androgen levels have been limited; most have relied on serum testosterone measurements in the setting of castration as a surrogate for prostatic androgen
levels, an assumption now known to be inaccurate. We hypothesized that despite castrate levels of serum testosterone, the degree and uniformity of androgen deprivation in the tumor microenvironment may be inadequate to consistently ablate androgen activity, as assessed by androgen-dependent gene expression. In this setting, incomplete suppression of tumoral androgen activity may lead to adaptive cellular changes, allowing tumor survival and proliferation in a low androgen environment. This scenario could explain the near-universal emergence of castration-resistant disease and offers an alternative to theories that postulate the existence of rare androgen-independent neoplastic clones early in tumorigenesis, or a requirement for genetic mutations producing selective growth advantages.

To determine the effect of medical castration on the intra-prostatic androgen milieu, we evaluated the effects of both short-term and prolonged androgen suppression on prostatic androgen-responsive gene and protein expression. We did a randomized clinical trial of short-term castration in healthy men and obtained prostate core biopsies to measure tissue androgen levels and epithelial cell gene expression. To determine the efficacy and uniformity of standard ADT in ablatting androgen-dependent gene expression in neoplastic tissue, we also evaluated the suppression of androgen-regulated gene and protein expression in primary prostate cancer specimens from men with localized prostate cancer treated with neoadjuvant ADT for up to 9 months.

**Materials and Methods**

**Clinical Protocols**

**Healthy men.** Prostate tissue samples were obtained from healthy subjects enrolled in a clinical trial of medical castration using the gonadotropin-releasing hormone antagonist Acyline (NeoMDS; ref, 10) done at the University of Washington (Seattle, WA; ref, 5). All procedures involving human subjects for this protocol were approved by the institutional review board of the University of Washington, and all subjects signed written informed consent. In brief, 12 healthy men (ages 35–55 years) with normal prostate size by digital rectal examination, serum prostate-specific antigen (PSA) of <2.0 ng/mL, and without known prostate disease signed written informed consent. In brief, 5-mm frozen tissue sections were stained using an avidin-biotin-peroxidase complex as the tertiary reagent (Vector Labs) and diaminobenzidine (DAB) as the chromogen. Images were made at ×40 and digitized with a SPOT Insight camera (model 3.2.0, Diagnostics Instruments, Inc.), using the same light intensity and color thresholding for all sections of each stain. Image analysis was done using Image Pro-Plus version 4.2 software (Media Cybernetics, Inc.). The percentage of total epithelial area (for PSA) or nuclear area (for AR) positively stained was assessed in a minimum of five glands and/or 500 epithelial cells. Successive tabulations were accumulated until the marginal change in the coefficient of variation of the running mean value (for the percentage area positively stained) was <5% (15). Treatment-related atrophy was evaluated in a blinded fashion by a genitourinary pathologist with expertise in identifying castration-related atrophic tissue changes (L.D.T.), using recently published criteria for classifying atrophy (16). Epithelial cell height was evaluated from the mean of nine height measurements per sample, based on measuring the vertical axis of the three shortest epithelial cells in each of three randomly selected ×40 fields using the measurement tool in Adobe Photoshop (Adobe Systems Incorporated). Images tying the length of an entire H&E-stained core biopsy were made at ×10 (10–12 images per core); the number of epithelial cells per unit volume was estimated by counting the number of nuclei within epithelial glands over the area of the biopsy and thickness of the section. Cancer specimens from men with different durations of ADT before prostatectomy were used to construct tissue microarrays for immunohistochemical analysis, as previously described (11, 12). In brief, the tissue microarray was created from three 0.6-mm cancer-containing cores from the radical prostatectomy specimen of each patient. The treatment groups were as follows: 0 months, n = 21; <3 months, n = 21; 3 to 6 months, n = 28; and 6 to 9 months, n = 22. Antigen retrieval from formalin-fixed and paraffin-embedded tissue sections was done by incubation with EDTA (pH 8) at 95°C for 8 min. Staining for PSA and AR was done using a Ventana autostainer, model Discover XT (Ventana Medical System), with an enzyme-labeled biotin streptavidin system and solvent-resistant DAB Map kit (Pierce). The staining intensity of malignant tissue was rated on a scale from 0 to 3, representing the range from no staining to heavy staining. The final score for each core was calculated from the sum of the percentage of positive cells at each intensity level, and the final score for each patient was calculated as the mean score over the three cores.

**Gene Expression Assays**

**Laser capture microdissection and RNA amplification.** Prostate samples embedded in OCT were used for laser capture microdissection (LCM). Approximately 2,000 to 3,000 epithelial cells per sample were collected from 8-µm sections using the Arcturus Veritas Laser Capture Microdissection System according to the Arcturus HistoGene LCM Frozen Section Staining Kit protocol. Total RNA was isolated using the Arcturus Picopure kit, and the samples were treated with DNase using the Qiagen RNase-Free DNase Set (Qiagen, Inc.). Total RNA was subjected to two for RNA analyses, as well as formalin fixed and paraffin embedded for immunohistochemical analyses. Prostate tissue from a randomly selected subset of patients at each time point (n = 3 per group, at 0, 3–6, and 6–9 months of treatment) were used to assess gene and protein expression.

**PSA and Hormone Measurements**

Serum PSA was measured in the Department of Laboratory Medicine, University of Washington, using a Hybritech Enzyme Immunoassay with inter- and intra-assay coefficient of variations of 2% to 4%. Serum and prostate testosterone and dihydrotestosterone levels were measured in the Endocrine Services Laboratory of the Oregon National Primate Research Center as previously described (5, 13). In brief, samples were extracted with diethyl ether and fractionated by liquid chromatography using hexane/ benzene/methanol, with assay of testosterone and dihydrotestosterone concentrations in the appropriate fractions by RIA (14).

**Histology and Immunohistochemistry**

Immunohistochemical staining and image analysis of frozen tissue sections from the Acyline trial for PSA (polyclonal anti-PSA, DAKO, Inc.) and AR (monoclonal anti-AR, clone F36.4.1, BioGenex) was done as recently described (5). In brief, 5-µm frozen tissue sections were stained using an avidin-biotin-peroxidase complex as the tertiary reagent (Vector Labs) and diaminobenzidine (DAB) as the chromogen. Images were made at ×40 and digitized with a SPOT Insight camera (model 3.2.0, Diagnostics Instruments, Inc.), using the same light intensity and color thresholding for all sections of each stain. Image analysis was done using Image Pro-Plus version 4.2 software (Media Cybernetics, Inc.). The percentage of total epithelial area (for PSA) or nuclear area (for AR) positively stained was assessed in a minimum of five glands and/or 500 epithelial cells. 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rounds of linear amplification using the Ambion MessageAmpII Kit (Ambion, Inc.), quantitated in a Gene-Spec III spectrophotometer (Hitachi) and aRNA integrity was evaluated using gel electrophoresis. RNA was extracted from microdissected benign epithelial cells from all subjects in the Acyline trial (placebo, castrate, and castrate + testosterone; \( n = 4 \) in each group), and from microdissected malignant epithelial cells from a randomly selected subset of patients in the neoadjuvant ADT trial (at 0, 3–6, and 6–9 months of treatment; \( n = 3 \) in each group).

cDNA microarray hybridization and analysis. cDNA probe pairs were prepared from samples obtained in the Acyline trial by amino-allyl reverse transcription using 2 \( \mu \)g of amplified RNA from the microdissected samples and 2 \( \mu \)g of amplified RNA from a benign prostate reference standard. The reference was created by pooling equal amounts of RNA amplified from the microdissected placebo-treated prostate epithelial samples. Samples from the neoadjuvant trial of ADT were prepared using 2 \( \mu \)g of amplified RNA from the microdissected prostate samples and 30 \( \mu \)g of total RNA from a reference RNA pool composed of total RNA isolated from LNCaP, DU145, and PC3 prostate cancer cell lines. Probes were labeled with either Cy5 or Cy3 fluors (Amersham Bioscience) and competitively hybridized to custom cDNA microarrays spotted in duplicate with ~6,700 unique cDNA clones from the Prostate Expression Database as previously described (17, 18).

Fluorescence array images were collected using a GenePix 4000B fluorescent scanner (Axon Instruments) and processed as we have previously described (19). Changes in gene expression were evaluated using the Statistical Analysis of Microarray program\(^8\) to perform a one-sample \( t \) test assessing the effect of Acyline treatment on gene expression (20). A false discovery rate (FDR) of \(<5\%\) was considered significant. Quantile normalization was done in Bioconductor (21) to allow single-channel comparison of the absolute signal intensities in the experimental channel across different arrays in the same experiment.

Quantitative reverse transcription-PCR. To further evaluate the results of the gene expression microarrays, amplified RNA was evaluated by quantitative PCR (qPCR). cDNA was generated from each sample using 0.5 \( \mu \)g of amplified RNA in a random hexamer-primed reverse transcription reaction. qPCR reactions were done in triplicate using an Applied Biosystems 7700 sequence detector with ~5 ng of cDNA, 1 \( \mu \)mol/L of each primer pair, and SYBR Green PCR master mix (Applied Biosystems). The mean cycle threshold (\( C_t \)) obtained for each gene was normalized to the expression of a housekeeping gene, \( RPL13A \), in the same sample. Primers specific for the genes of interest were designed using the Web-based primer design service Primer3 provided by the Whitehead Institute for Biomedical Research.\(^9\) The sequences of primers used are provided in the supplemental data (Supplementary Table S1).

Results

Prostate epithelial cell gene expression changes after short-term castration in normal men. Compared with placebo-treated men, medical castration using the gonadotropin-releasing hormone antagonist, Acyline, decreased mean serum testosterone to castrate levels throughout the 1 month treatment period (day 28 values \( \pm \) SE: placebo, 357 \( \pm \) 86 ng/dL versus castrate, 26 \( \pm \) 9 ng/dL) and reduced mean prostatic androgen levels by \( \sim70\% \) to \( \sim80\% \), as we previously reported (5). Androgen levels for each individual at the time of prostate biopsy (day 28) are reported here (Table 1) to facilitate comparison with gene expression measurements. Microarray analysis of prostate epithelial cell transcripts isolated by LCM showed differential gene expression in the castrate versus placebo-treated men (Fig. 1), with 83

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\(^8\) http://www-stat.stanford.edu/~tibs/SAM/index.html

unique genes down-regulated and 106 genes up-regulated by $\geq1.5$-fold (FDR 5%). Several of the genes most strongly down-regulated after castration (e.g., NDRG1, FKBP5, and TMPRSS2) have previously been shown to exhibit substantial transcript level changes in LNCaP cells treated with androgens (22). Androgen deprivation altered the expression of genes related to transcription, proliferation, and apoptosis; carbohydrate and sterol metabolism; cellular structure and adhesion; transport and trafficking pathways; and inflammatory and stress responses (Supplementary Table S2).

Notably, despite castrate serum testosterone levels and an average 75% reduction in tissue androgen levels, the transcript levels of many genes previously reported as androgen-regulated (AR, PSA, ACPP, NKX3.1, seladin, KLK2, and TMEPAI) were not statistically differentially altered in the castrate subjects. As PSA is one of the most abundant transcripts in normal prostate secretory

Figure 1. Differential gene expression in microdissected prostate epithelial samples from castrate (Acyline treated) versus placebo subjects. The top 25 genes up- or down-regulated by castration ($>1.5$-fold with false-discovery rates $<5$%) are depicted on a gray scale representing the fold change relative to the placebo reference standard. White, down-regulation; gray, no regulation; black, up-regulation. Genes in bold face, genes previously identified as androgen responsive.

Figure 2. qRT-PCR evaluation of microarray data. A, the mean fold change in transcript expression induced by castration (Acyline) relative to placebo as determined by qRT-PCR. The cycle threshold ($C_t$) for each gene was normalized to the housekeeping gene RPL13A. Two-sample $t$ tests were used to compare the difference in mean $C_t$ values between the castrate and placebo-treated samples. $P$ values $<0.05$ in castrate versus placebo samples (bold print) were considered significant. The fold change was calculated by unlogging the difference in $C_t$ between the treatment groups. Middle column, fold changes for genes that were differentially regulated on the microarray are shown for comparison. B, qRT-PCR results for selected androgen-influenced genes of interest showing heterogeneity in transcript expression among individual subjects. Y axis, fold change in transcript expression (relative to RPL13A). Horizontal bars, means. $\pm$, the castrate subject with the lowest tissue androgen levels (Acyline subject 109).
epithelial cells, we further examined the microarray data to determine how castration affected both the absolute and rank order of PSA expression. Interestingly, the rank order of PSA expression varied substantially among the castrate subjects, being marginally lowered in two subjects and only markedly lowered in one, compared with placebo-treated controls (Table 1). Quantile normalization of the array data, to allow single-channel comparison of the absolute signal intensities, showed ∼10-fold lower PSA signal intensity in the sample from the castrate subject that had the greatest decrement in PSA rank (sample 109, rank 895), but remained among the 50 most abundant transcripts in the remaining three individuals (nos. 3, 45, and 17; Table 1). That PSA was not statistically identified as differentially regulated in the microarray analysis reflects the dependence of the Statistical Analysis of Microarray algorithm on both the magnitude and consistency of change across a data set to meet criteria for significance. Interestingly, the castrate subject (no. 109) in whom the most marked individual alterations in gene expression were observed had the most profoundly suppressed tissue androgen levels, with a prostatic dihydrotestosterone level three to five times lower than that of other subjects in the castrate cohort (Table 1). Notably, he did not have the lowest serum androgen level among the castrate subjects.

The microarray findings were verified by performing quantitative reverse transcription-PCR (qRT-PCR) for a selected set of genes that were either among those highly regulated on the microarray analysis, or known androgen-responsive genes not identified as differentially regulated by microarray measurements (e.g., AR, PSA, ACPP, and NKX3.1; Fig. 2A). Among the 10 genes identified as differentially regulated on the array that were tested by qRT-PCR, statistically significant fold changes between the castrate and placebo-treated subjects were confirmed for six genes (P < 0.05 for TFF1, FOLH1, FKBP5, NDRG1, IGFBP5, and MME), with P values for the remaining four genes trending toward significance. Results for the castrate + testosterone treatment group were essentially identical to the placebo cohort (data not shown).

Marked heterogeneity among the castrate subjects was observed for the remaining androgen-responsive genes evaluated by qRT-PCR (Fig. 2B). In the castrate cohort, the single subject exhibiting the substantially lower tissue dihydrotestosterone level and PSA rank (no. 109; Table 1) also showed the lowest expression levels of the other androgen-responsive genes. Interestingly, qRT-PCR analysis for clusterin, an antiapoptotic factor strongly up-regulated in LNCaP cells and other androgen-sensitive tumor models after androgen deprivation (12, 23), was also markedly higher only in that particular subject, whereas it was unchanged in samples from the remaining castrate subjects (Fig. 2B).

Immunohistochemical staining for PSA and AR paralleled the changes in transcript expression (Fig. 3A and B). As we have previously reported, the castrate subjects did not, as a group, exhibit alterations in the mean level of staining for AR or PSA (5). However, as is shown in the quantitative immunohistochemistry scores for each subject, staining for both PSA (Fig. 3C) and AR (Fig. 3D) was markedly decreased in subject no. 109 compared with both the placebo controls and with other subjects in the castrate group. As discussed in our recent report, mean serum PSA levels in the castrate cohort did show a statistically significant decline (5). However, despite having the lowest tissue expression of PSA, patient 109 in the castrate group did not have the largest absolute or relative decline in serum PSA (data not shown). These findings are consistent with previous observations showing a discrepancy between tissue and serum levels of PSA (24, 25).

To more fully evaluate the cellular androgen context for these gene expression changes, image analysis was used to determine prostatic androgen levels on a per-epithelial-cell basis. As androgen levels may be higher in epithelial cells compared with stroma (26), the androgen levels per epithelial cell (and therefore androgen-regulated gene expression) might be preserved if castration decreased the epithelial cell compartment (27). In this regard, an increase in epithelial cell apoptosis has been observed at 3 to 4 days after castration (28). Alternatively, castration might result in a relative increase in epithelial cells if shrinkage of the stromal

Figure 3. Immunohistochemical analysis of frozen biopsy samples from the Acyline trial. Staining results for two representative placebo samples and all four castrate samples for PSA (A) and AR (B). Magnification, ×40. Arrows, areas of decreased PSA and AR expression in epithelial tissue of the castrate subject with the lowest tissue androgen levels (Acy-109). Quantification of immunohistochemical staining for PSA (C) and AR (D) was done using Image Pro Plus image analysis software. Positive staining was quantified based on the percentage of total gland area for PSA, and the percentage of total nuclear area for AR. Horizontal bars, mean. Filled symbols, individual results for subjects in each treatment group; △, castrate (Acyline) subject no. 109.
component predominates. However, we found the mean number of epithelial cells per gram to be similar in the castrate and placebo subjects (3.33E+08 and 3.2E+08, respectively), such that the relative decrease in hormone levels between the two groups (as reported per gram of prostate) was preserved (Supplementary Table S3). Of note, at 1 month of castration, we did not observe significant changes in prostate size (5), or consistent changes in epithelial cell height (Supplementary Table S3); we also did not see any marked features of atrophy in the prostate epithelial cells, including those features that have been reported in studies of longer duration androgen deprivation (27).

Androgen-regulated gene expression in primary prostate tumors after prolonged neoadjuvant androgen suppression.

To evaluate the efficacy of prolonged androgen deprivation in suppressing androgen-regulated gene expression in prostate tumor cells, we examined prostate tumors obtained via prostatectomy from men treated with up to 9 months of neoadjuvant hormonal deprivation. These also showed a heterogeneous and incomplete suppression of androgen-regulated genes, even at the longest time points of therapy.

Transcript and protein expression was evaluated in microdissected prostate cancer specimens from a subset of nine patients at three time points after androgen deprivation (0, 3–6 and, >6–9 months; n = 3 in each group). The rank order of PSA transcript abundance in each patient was evaluated using microarray hybridization (Fig. 4A), and was similar in patients at 0 months of treatment (rank order range 3–7) to that seen in prostatectomy subjects (rank order range 1–5) in the Acyline trial. PSA transcript abundance was marginally but consistently suppressed in samples from the neoadjuvant-treated patients at 3 to 9 months of therapy. However, one patient in the 3–6-month treatment group actually showed a PSA abundance level (rank 7) comparable with untreated patients, and the lowest rank order of PSA expression achieved was only 37 in the androgen-suppressed patients.

The effect of prolonged androgen blockade was further explored by evaluating the expression of six known androgen-responsive genes by qRT-PCR. For each gene, differences in transcript abundance in each patient was evaluated using microarray hybridization. The expression of these genes despite prolonged androgen suppression. Consistent with numerous reports identifying the sensitivity of FKBP5 to androgen manipulation (29), FKBP5 was the only gene statistically down regulated between the 0- versus 3- to 9-month treatment groups (P = 0.017).

To confirm gene expression measurements at the protein level, the expression of PSA and AR was examined by immunohistochemistry, using a tissue microarray comprising prostate tumor samples from 94 patients treated with varying durations (0, 3, 6, and 9 months) of androgen suppression (Fig. 5). The expression of both PSA (Fig. 5A) and AR (Fig. 5B) in prostate cancer was markedly heterogeneous at all time points of therapy. PSA expression was statistically significantly decreased in tissue cores from patients at 0 months versus 3 to 6 months of therapy [mean PSA score at 0 months 2.2 (95% confidence interval, 95% CI, 1.9–2.5) versus 1.4 (95% CI 1.3–1.6) at 3–6 months; P < 0.001]. However, expression was by no means eliminated, remaining detectable in all cancer cores, and the mean PSA score at 6 to 9 months (1.8; 95% CI, 1.5–2.1) was similar to that observed at 1 to 3 months of therapy (1.8; 95% CI, 1.4–2.1). Importantly, at each time point, cells expressing PSA in each case were not rare, but usually represented the predominant population of cancer cells. AR staining was heterogeneously present at all time points and was neither more strongly nor more consistently suppressed with longer duration of treatment, even at 6 to 9 months of therapy. In contrast to these histologic observations, marked decreases in serum PSA levels at each time point of androgen deprivation were measured, with the average serum PSA level declining from 5.7 to 2.3, 0.4, and 0.6 ng/dL at 0, <3, 3 to 6, and >6 months of treatment, respectively. However, nearly two thirds of patients at the longest time point of therapy still had detectable serum PSA levels (>0.1 ng/dL).

Discussion

In this study, we show that the serum and prostatic androgen levels achieved by standard medical castration are inadequate to effectively suppress prostatic androgen–regulated gene expression. We observed persistent expression of androgen-regulated genes in benign prostate epithelial samples from healthy men.
Hormonal deprivation also showed that androgen signaling was substantially more thorough inhibition of prostatic androgen activity observed in the neoadjuvant trial might arguably reflect the activity. The generally limited suppression of androgen-regulated levels that were three to five times lower than that of the other subects in the cohort. Although obviously of limited significance, this observation begins to suggest that achieving more castrate subjects in the cohort. Although obviously of limited significance, this observation begins to suggest that achieving more castrate subjects in the cohort.

Figure 5. Immunohistochemical analysis of samples from the neoadjuvant trial of androgen suppression. Staining for PSA (A) and AR (B) was evaluated in a tissue microarray composed of cancer containing cores from patients at 0 mo (n = 21), <3 mo (n = 21), 3 to 6 mo (n = 28), and 6 to 9 mo (n = 22) of treatment. The intensity of staining and the percentage of positively stained tumor cells were each evaluated. Y axis, overall score, derived by combining the percentage of positively stained cells at each intensity level. Horizontal bars, mean. Differences among the four treatment groups were compared by one-way ANOVA with Bonferroni’s correction for multiple testing. PSA expression was statistically significantly decreased in tissue cores from patients at 0 mo versus 3 to 6 mo of therapy (*P < 0.001). No other changes were statistically significant.

after short-term castration, as well as in neoplastic prostate tissue from men with localized prostate cancer after prolonged neoadjuvant hormonal suppression. Moreover, PSA and AR expression were widely detectable by immunohistochemistry in both benign and neoplastic tissue after castration, and remained so in the neoplastic samples examined after up to 9 months of androgen suppression.

Of interest, the only subject in the Acyline trial of medical castration in whom marked suppression of prostatic androgen-responsive genes was observed had tissue dihydrotestosterone levels that were three to five times lower than that of the other castrate subjects in the cohort. Although obviously of limited significance, this observation begins to suggest that achieving more potent suppression of tissue androgen levels may result in substantially more thorough inhibition of prostatic androgen activity. The generally limited suppression of androgen-regulated gene activity observed in the Acyline trial might arguably reflect the relatively short duration of androgen deprivation to which subjects were exposed; moreover, observations in benign tissue may not necessarily reflect those in neoplastic tissue (30). In this regard, the temporal analyses of androgen-regulated genes in primary prostate tumors from men treated with up to 9 months of neoadjuvant hormonal deprivation also showed that androgen signaling was maintained at each time point evaluated, although significant heterogeneity was evident. As in a previous report by Holzbeierlein et al. (31), we also observed an effect on the expression of androgen-regulated genes in a number of the neoadjuvant samples examined by qRT-PCR (although not reaching statistical significance as a group). However, our interpretation of these results emphasizes the findings of continued substantial androgen-dependent gene expression in most cases, as well as the heterogeneity of the treatment response to ADT, rather than a uniform inhibition of androgen-influenced transcription. Our study has several limitations, including relatively small numbers of subjects in which RNA expression was evaluated, the short duration of castration in the Acyline trial, and the lack of tissue androgen measurements in the neoadjuvant trial. Given the heterogeneity in gene expression, larger numbers of samples would clearly be necessary to show whether additional statistically significant fold changes in gene expression occurred due to castration.

Although androgen suppression remains the primary treatment modality for patients with advanced prostate cancer, interpreting the clinical efficacy of androgen deprivation strategies is hampered by a reliance on the serum testosterone level as a surrogate indicator of prostatic and/or tumoral androgen concentrations. Moreover, substantial evidence suggests that castration-resistant prostate cancer may not, in fact, be androgen independent, but occurs in a setting of continued AR-mediated signaling that is driven by the presence of residual tissue androgens (32). That ~30% of recurrent prostate tumors show at least transient clinical responses to secondary or tertiary hormonal manipulation provides support for this conclusion (33, 34). Furthermore, analyses of serum and tissue samples obtained in the setting of tumor progression in clinically castrate patients have shown the persistent or recurrent expression of androgen-regulated genes, including measurable serum levels of PSA (3, 31). Our findings provide evidence that benign prostate samples obtained from healthy men, as well as tumor samples obtained from men with localized prostate cancer before disease progression, also show ongoing androgen-regulated gene expression in the setting of medical castration. Androgen suppression therapy resulted in a significant decline in serum PSA concentrations despite persistent tissue PSA expression in normal subjects after short-term castration as well as in prostate cancer patients after prolonged androgen deprivation. This finding is consistent with prior observations showing a discrepancy between serum PSA levels and tissue PSA expression (24, 25), and serves as a reminder that changes in serum PSA concentrations are not necessarily an accurate surrogate for tissue response. Because androgens influence prostatic vasculature (35–37), the decline in serum PSA may reflect changes in tissue vascularity and PSA leak (38).

Defining the mechanisms responsible for the emergence of castration-resistant prostate cancer has important clinical ramifications. Intensive basic and clinical research efforts have identified several potential explanations that include increased sensitivity of the AR via gene amplification and/or overexpression (39, 40), alterations in coregulators that alter ligand sensitivity (41), AR mutations that broaden ligand specificity and confer sensitivity to adrenal androgens (42), cross-talk via other signaling pathways such as IL-6 and Her2-Neu (43, 44), nongenomic mechanisms by which ligand-bound AR modulates transcription (45), and alterations in steroid synthetic and androgen-metabolizing enzymes that potentiate intraprostatic androgen production (31, 46–48).
Importantly, most of these cellular alterations still require the presence of some, albeit lower, androgen concentrations.

The findings reported here also provide support for the idea of therapy-mediated selection pressure. Specifically, suboptimal reduction of tumoral androgen activity may contribute to the outgrowth of resistant prostate cancer cells adapted to survive in a low androgen environment (7), suggesting that markedly more effective methods for suppressing the androgen axis in the tumoral microenvironment are required. The key feature of this explanation involves a process of metabolic adaptation whereby alterations in cellular components occur through transcriptional or translational mechanisms rather than genomic events such as mutation, translocation, or the amplification of chromosomal loci. These latter events would be expected to occur rarely in a population of tumor cells and to subsequently dominate through a selective growth advantage, such that an increasing number of clones expressing androgen-responsive genes would be evident over time. Conversely, we have observed that large numbers of spatially distinct tumor cells express androgen-regulated genes at all time points evaluated after castration. These findings suggest that a more generalized adaptive response to a low-androgen environment is occurring. For example, the enhanced production or activity of enzymes responsible for converting adrenal androgens to testosterone could contribute to castration-adapted growth, as has been measured in advanced metastatic prostate cancers (49).

In summary, we have shown that the reduction of circulating androgens to castrate levels and interruption of AR signaling by androgen deprivation are neither reliably effective nor uniform in suppressing prostatic androgen-regulated gene and protein expression. Our findings suggest that medical castration based on serum testosterone levels cannot be equated with ablation of androgen levels or androgen-mediated activity in the prostate tissue microenvironment. These findings underscore the need for demonstrating suppression at the target tissue and molecular level before drawing conclusions regarding the clinical efficacy of hormonal treatment strategies. Importantly, the suboptimal suppression of tumoral androgen activity may account for the heterogeneity in treatment effect observed across individual patients, and, moreover, may contribute to the outgrowth of resistant prostate cancer clones adapted to survive in a low androgen environment. Optimal clinical efficacy will require testing of novel approaches targeting complete suppression of testicular, adrenal, and intracrine contributions to the prostatic androgen milieu.

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References

prostate gene expression after castration


