The Adrenal Androgen Androstenediol Is Present in Prostate Cancer Tissue after Androgen Deprivation Therapy and Activates Mutated Androgen Receptor

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ABSTRACT

Despite an initial response to androgen deprivation therapy, prostate cancer (PCa) progresses eventually from an androgen-dependent to an androgen-independent phenotype. One of the mechanisms of relapse is antiandrogen withdrawal phenomenon caused by mutation of 877th amino acid of androgen receptor (AR). In the present study, we established a method to measure the concentration of androstenediol (adiol) in prostate tissue. We found that adiol maintains a high concentration in PCa tissue even after androgen deprivation therapy. Furthermore, adiol is a stronger activator of mutant AR in LNCaP PCa cells and induces more cell proliferation, prostate-specific antigen (PSA) mRNA expression, and PSA promoter than dihydrotestosterone (DHT). Because antiandrogen, bicalutamide, blocked adiol activity in LNCaP cells, it was suggested that adiol effect was mediated through AR. However, high concentration of bicalutamide was necessary to block completely adiol activity. These effects were specific to LNCaP cells because adiol had less effect in PC-3 PCa cells transfected with wild-type AR than DHT and had similar effect in PC-3 cells transfected with mutant AR. The mechanism that adiol activates mutant AR in LNCaP cells did not result from the increased affinity to mutant AR or from AR's association with coactivator ARA70. However, low concentration of adiol induced more AR nuclear translocation than DHT in LNCaP cells and not PC-3 cells transfected with AR. These results indicate that adiol may cause the progression of PCa even after hormone therapy.

INTRODUCTION

Prostate cancer (PCa) is the most common malignancy and the second most frequent cause of cancer-related death of men in the United States (1). Because PCs depends initially on androgens for growth, androgen deprivation therapies (usually some combination of aneuctirome, luteinizing hormone-releasing hormone analogues, and antiandrogens) often are the first choice of several therapeutic procedures for advanced PCs. The initial response rate to androgen deprivation therapy is 80–90%. However, the cancer reoccurs eventually and is considered to be androgen independent. The molecular mechanisms that account for the development to apparent androgen independence after the initiation of androgen deprivation therapy remain unclear. The observation that heterogeneity of androgen receptor (AR) expression in PCa correlates with malignancy suggests that androgen responsiveness plays an important key role in the progression of PCa (2).

Dehydroepiandrosterone (DHEA) accounts for the majority of androgen secreted from the adrenal gland. DHEA is converted in several cell types, including prostate epithelial cells, to androst-5-ene-3,17β-diol (3β,17β-diol [Δ3-androstenediol (adiol)]). The concentration of adiol in plasma is ~1.5 ng/ml (5.2 × 10⁻⁹ M). Orchiectomy reduces the plasma concentration of testosterone (T) by >90%, but it reduces adiol plasma concentration by only 50% (3). Although we have identified that adiol is the major metabolite of DHEA in human prostate homogenate in vitro (4), adiol levels in PCa tissues postorchectomy have not been reported. This may be important to the progression of PCa because in addition to being converted to T and dihydrotestosterone (DHT), which activate the AR, adiol itself activates the AR (5).

On the basis of adiol’s ability to activate the AR and its presence in prostate tissues, we explored the hypothesis that adiol provides androgenic stimulation of PCs even after androgen deprivation therapy. In the present study, we demonstrate that although DHT levels decrease, adiol levels are unchanged in PCa tissues after androgen deprivation therapy. Furthermore, we show that adiol is stronger than DHT at activating a mutated AR in a PCa cell line.

MATERIALS AND METHODS

Quantitative Analysis of Adiol and DHT in the Prostate. Benign prostatic hypertrophy tissues without any hormonal treatments (n = 15) and PCa tissues with neoadjuvant hormone therapies for 3–6 months before radical prostatectomy (n = 12) were obtained from patients, who gave informed consent at Kanazawa University Hospital. Neoadjuvant hormone therapies were combinations of luteinizing hormone-releasing hormone agonist and antiandrogen drugs [chloromadinone acetate (two patients), hydroxyflutamide (seven patients), estramustine phosphate (one patient), or bicalutamide (two patients)]. The steroid concentration in prostate tissues was measured according to Shibata et al. (6). The powdered prostate was weighed for estimation of the tissue amount used for the analysis and then homogenized in 3 ml of distilled water using an Ultra-Turrax homogenizer (IKA, Staufen, Germany) on ice. Five ng of androstenediol-3H2, 5 ng of T-19-C14H, and 5 ng of [17,16,16-3H3]-DHT were added as internal standards to the individual homogenized sample, which then was extracted with 20 ml of 40% ethylacetate/n-hexane twice. The collected organic layer was washed with 5% NaHCO3 and distilled water and then dried over anhydrous Na2SO4. The extracts were dissolved after evaporation in 1 ml of 30% acetonitrile/H2O in an ultrasonic bath and applied to a 3-ml Bond Elut C18 cartridge column (Varian, Harbor City, CA). These columns then were washed with 3 ml of 30% acetonitrile/H2O, and the steroid fraction was eluted with 2.5 ml of 70% acetonitrile/H2O and dried using a centrifugal evaporator. The extract was divided into adiol fraction, T, and DHT fraction, respectively. The dried DHT and T fractions were reacted with 100 μl of carboxymethoxylamine hemihydrochloride solution (carboxymethoxylamine hemihydrochloride in pyridine, 2 mg/ml) overnight in the dark at room temperature. The carboxymethoxylamine hemihydrochloride derivatives in 0.02 N HCl solution were applied to a 3-ml Bond Elut C18 cartridge column. The columns were washed successively with 3 ml of distilled water and 6 ml of methanol/0.5% acetic acid (1:1), and the steroidal fraction then was eluted with 3 ml of methanol/H2O (2:1). The collected fraction was evaporated to dryness; the obtained samples were dissolved in 100 μl of high-performance liquid chromatography solvent; and 50 μl was applied to a liquid chromatography-tandem mass spectrometry (LC-MS/MS) instrument as described earlier. For DHT, the molecular ion mass of 364 m/z was cleaved again, and the produced ion mass of 270 m/z was monitored. The produced ion mass monitored for internal standards T-19-C14H and [17,16,16-3H3]-DHT were 271 and 273 m/z, respectively. The assay was validated to ensure that the result was within the 20% range of accuracy and precision. The lower limit values were 65 pg for DHT and T. Adiol fraction was treated with pyridine (50 μl) and acetic anhydride (50 μl) overnight at room temperature.
After evaporation of the solution, the sample was treated in 100 µl of high-performance liquid chromatography solvent, and 50 µl was applied to an LC-MS/MS instrument. The product ion, 255.2 m/z (250.2 m/z for the internal standard), was monitored for determination. The assay was validated to ensure that the result was within the 20% range of accuracy and precision. The lower limit values were 300 pg for adiabicate.

The plasma T level was estimated by LC-MS/MS using T-3H, as an internal standard. The plasma (100 µl) that involved in an internal standard (1 ng) was extracted with ether, followed by treatment with 1-methyl-2-fluoropyridine to T-17-O-1'-methylpyridinium derivative. After the derivatization was purified on a cartridge column, a part of the derivative was applied to an LC-MS/MS. Analytical ions were used with 253 m/z and 256 m/z product from the molecular ions, respectively. The determination lower limit was 10 pg (50 pg/ml) for T. The plasma adiolo level also was estimated by LC-MS/MS as described earlier.

Cell Culture and Cell Proliferation Assay. LNCaP cells were cultured in DMEM containing 5% FCS and penicillin/streptomycin (Invitrogen, Carlsbad, CA). Cell proliferation assay was performed by plating 1 × 10⁶ cells on six-well plates in DMEM-5% charcoal-stripped FCS. Twenty-four h later, cells were treated with the indicated concentration of T, DHEA, DHT, androstenedione (4-dione), or adiolo (Sigma, St. Louis, MO) and cultured for an additional 4 days. Media and reagents were replaced after 2 days. At the end of the culture period, the cells were trypsinized and counted with a hemocytometer.

RNA Extraction and Reverse Transcription-PCR. Twenty-four h after plating of 5 × 10⁵ LNCaP cells, cells were treated with the indicated concentration of DHEA, DHT, 4-dione, or adiolo for 12 h. Total RNA was purified with RNeasy mini kit (Qiagen, Valencia, CA). cDNA was made by reverse transcription of 1 µg of each total RNA using ThermoScript reverse transcription-PCR system (Invitrogen). Each cDNA sample was amplified with ExTaQ (Takara, Tokyo, Japan). PCR reaction for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and prostate-specific antigen (PSA; 94 °C for 15 s, 56 °C for 30 s, and 72 °C for 30 s) was carried out using the following forward and reverse primers to detect GAPDH and PSA. The primer pairs were PSA-forward, 5'-GCTTCTCCGAGCCAGGCTC-3'; PSA-reverse, 5'-GAGGACTTGGGAC-3'; GAPDH-forward, 5'-GACCACAGCTCATGC-3'; and GAPDH-reverse, 5'-TCCAACACCTGGTTGTA-3'. Each of the amplified products for PSA and GAPDH was determined by electrophoresis on a 1.5% agarose gel.

Scachard Analysis. [1,2,4,5,6,7-3H]-5α-DHT (specific activity 4007 Gbq/mmol) was obtained from New England Nuclear (Boston, MA). [1,2,6,7-3H]-androst-5-ene-3β, 17β-diol(3H, Δ5-adiolo) (specific activity 2220 Gbq/mmol) was synthesized from [1,2,6,7-3H]-DHEA by reduction of NaBH₄ in methanol at 4°C and then purified by liquid chromatography. Radiolabeled DHT and adiolo were obtained from Steraloids Inc. (Wilton, NH). Confluent cultures were serum-starved in ice-cold hypotonic buffer (300 mM KC1, 10 mM Tris, 1.5 mM EDTA, 5 mM DTT, and 10 mM sodium molybdate, (pH 7.4)), and disrupted by sonication. The soluble extracts were prepared by ultracentrifugation at 105,000 × g for 30 min at 4°C. Scachard analysis for DHT and adiolo were determined based on the data from total binding and nonspecific binding assay. For total binding, extracts (250 µl) were incubated in duplicate with seven steps of increasing concentrations of [3H]-DHT (0.05–3.65 nm) or [1H]adiolo (0.058–3.7 nm) for 18 h. Nonspecific binding was carried out by addition of 250-fold excess unlabeled T and adiolo, respectively, to the hot tubes including seven steps of increasing concentration of each labeled compound. Bound and free hormones were separated by the dextran-coated charcoal method described by Green and Leake (7). After incubating, 500 µl of dextran-coated charcoal (final concentration 0.25% charcoal, 0.025% dextrane-7-70 in buffer) were added to each assay tube for total binding or nonspecific binding centrifugation to pellet the charcoal at 1200 × g for 10 min, 0.5 ml supernatant of total and nonspecific binding tubes was assayed for radiolabeled binding to protein. Specific binding was calculated by subtracting nonspecific binding obtained in the presence of excess of T or adiolo from total binding measured in the absence of radiolabeled steroids.

Transfection and Luciferase Assay. Twenty-four h after plating 2 × 10⁵ cells on six-well plates in DMEM-5% charcoal-stripped FCS, LNCaP cells were transfected by calcium phosphate precipitation with 1 µg of luciferase reporter plasmid, pGL3PSa5.8, driven by 5.8 kb PSA promoter (9), 0.2 µg β-galactosidase expression plasmid, and 1 µg of pBluescript plasmid (Stratagene, La Jolla, CA) to serve as a carrier. For transfection of PC-3 cells, human AR expression plasmid, pCMV-AR or pCMV-ARmut877 (kindly provided by Chawshang Chang, University of Rochester, Rochester, NY), was cotransfected instead of pBluescript. Twenty-four h after transfection, the medium was changed, and cells were treated with the indicated concentration of T, DHEA, DHT, 4-dione, or adiolo. Cells were harvested 24 h after addition of reagents and lysed in luciferase lysis buffer (Promega, Madison, WI). The luciferase activity was quantitated by a microplate luminometer, and the results were normalized by β-galactosidase activity.

Western Blot Analysis. Twenty-four h after plating 1 × 10⁶ LNCaP or 7 × 10⁵ PC-3 cells on 6-cm dishes in DMEM-5% charcoal-stripped FCS, the cells were treated with indicated concentration of DHT or adiolo and harvested. After plating 7 × 10⁵ PC-3 cells, cells were transfected with 4 µg pCMV-AR or pCMV-Armut877 and cultured for 24 h. The cells then were treated with the indicated concentration of DHT or adiolo. The cells were lysed with 200 µl hypotonic buffer [20 mM Tris- HCl (pH 7.6), 10 mM NaCl, 1 mM MgCl₂, and 0.5% NP-40], and the cytosol fraction was removed by centrifugation. The precipitated nuclei were lysed with 100 µl hypotonic buffer [20 mM Tris-HCl (pH 7.6), 0.42 mM NaCl, and 1 mM EDTA], and nuclear protein was extracted. Fifteen µg of nuclear protein were loaded in each lane of an 8% SDS-polyacrylamide gel, subjected to electrophoresis, and then electrotransferred to a polyvinylidene difluoride membrane (Bio-Rad, Hercules, CA). The immobilized proteins were incubated with 5000-fold diluted antihuman androgen receptor antibody NH27 (8). The presence of NH27 was identified by 3000-fold diluted antirabbit antibody conjugated with alkaline phosphatase and stained by Immun-blot kit (Bio-Rad). The intensity of AR protein bands was quantitated by NIH image 1.61 after an image of the membrane was scanned into a Macintosh computer (Apple, Cupertino, CA).

Tumorigenesis of LNCaP Cells in Vivo. Thirty-six 6-week-old male severe combined immunodeficient mice were castrated 2 days before inoculation of cells. These mice were divided simultaneously into three groups. One group was a control group (n = 12). The other two groups were implanted s.c. with a silastic tablet containing 5 mg DHT (n = 12) or adiolo (n = 12). The 2 × 10⁵ cells/mouse were suspended in 50 µl of DMEM-10% FCS medium and 50 µl of Matrigel (BD Biosciences, San Jose, CA) and were inoculated s.c. into the right flank lesion on the treated mice using a 29-gauge needle. The tablets were implanted every 4 weeks. Tumor volume was measured every 5 days (length × width/2, length > width) as described by Fujita et al. (10). After measuring tumor size on day 50 of inoculation, all of the mice were killed; blood was taken for measuring T and adiolo concentration; and the weight of prostates and seminal vesicles was measured.

RESULTS

To explore the effect of decreased serum androgen levels on DHT and adiolo concentration in prostate tissues, trans-urethral resected benign prostate hypertrophy tissues from patients not receiving hormonal treatments and PCa tissues from patients receiving androgen deprivation therapy for 3–6 months before radical prostatectomy were subjected to liquid chromatography-mass spectrometry to measure DHT and adiolo levels (Fig. 1). DHT and adiolo concentrations in benign prostate hypertrophy tissue were 2.48 ± 0.57 and 2.33 ± 0.58 ng/g, respectively, whereas those in the PCa tissue were 0.619 ± 0.21 and 2.81 ± 0.59 ng/g tissue, respectively. Thus, although androgen deprivation therapy reduces DHT levels in PCa tissue, it does not impact on adiolo level. This finding is consistent with the possibility that adiolo in PCa tissue after hormone therapy contributes to PCa progression.

To determine whether adiolo has an androgenic effect on PCa cells, we compared the effect of adiolo to other DHEA metabolites on proliferation of androgen-sensitive LNCaP cells. LNCaP cells were cultured at the different concentrations of DHEA, T, DHT, 4-dione, or adiolo for 4 days. Adiolo induced the 20% higher LNCaP cell proliferation at a 10-fold lower concentration than T or DHT and between 100- and 1000-fold lower concentration than DHEA or 4-dione (Fig. 2A). Because it is well known that the production of PSA also is induced by androgen, we investigated expression level of PSA mRNA
in LNCaP cells treated with various androgens for 12 h (Fig. 2B and C). More than $10^{-9}$ M DHT increased PSA mRNA level, and 4-dione and DHEA hardly increased it. In contrast, even low adiol concentration ($10^{-11}$ M) increased PSA mRNA level to two to four times of control. These results suggest that adiol itself has the ability to induce LNCaP cell proliferation and PSA mRNA expression because low adiol concentration cannot be converted to high T or DHT concentration that stimulates proliferation. To evaluate further the direct ability of adiol as androgen, we transfected LNCaP cells with a luciferase reporter plasmid (pGLPSAp5.8) driven by 5.8 kb of the PSA promoter that includes androgen response elements and compared induction of the reporter by DHT, T, adiol, 4-dione, and DHEA. Adiol at $10^{-10}$ M induced PSA promoter activity by 28-fold as opposed to 2-fold by DHT or T at equimolar levels (Fig. 3A). Furthermore, $10^{-10}$ M 4-dione or DHEA hardly induced PSA promoter activity. This result, combined the ability of adiol to induce LNCaP cell proliferation and PSA production, suggests that adiol itself has strong androgenic activity in LNCaP cells.

To determine if adiol mediates its activity through the AR, we examined if an antiandrogen, bicalutamide, can block adiol’s ability to stimulate the PSA promoter. LNCaP cells were transfected with pGLPSAp5.8 and treated with DHT or adiol in the presence of various concentrations of bicalutamide. Bicalutamide blocked the activity of DHT and adiol in a dose-dependent manner, although a higher concentration of bicalutamide was necessary to block adiol (Fig. 3B). This result demonstrates that adiol’s ability to activate the PSA promoter requires the AR and that adiol has strong androgenic activity in LNCaP cells.

To ensure the results were not specific to LNCaP cells, we tested whether adiol is androgenic in the PC-3 cells. For this reason, we cotransfected wild-type AR expression plasmid (pCMV-AR) and the pGLPSAp5.8 reporter into PC-3 cells and compared the androgenic ability among DHT, 4-dione, and DHEA, and adiol. In contrast to the results using LNCaP cells, DHT induced greater PSA promoter activity than adiol in PC-3 cells (Fig. 4). Additionally, 4-dione had weak androgenic activity, and DHEA did not induce PSA promoter activity.

One difference that could account for the discordant results between the two cell lines is that the AR in the LNCaP cells is mutated (a mutation at the position of 877 amino acid), and we had transfected PC-3 cells with the wild-type AR (11). The 877 mutation has been shown to confer promiscuity of the AR’s response to steroids. Therefore, we postulated that the increased responsiveness to adiol in LNCaP cells compared with PC-3 cells transfected with wild-type AR was the result of the LNCaP cell’s mutated AR. To test this possibility, we cotransfected PC-3 cells with the pGLPSAp5.8 reporter and either a wild-type AR or a mutated AR expression plasmid (pCMV-ARmut877), which encodes the same AR mutation found in LNCaP cells, and compared DHT’s and adiol’s effects on PSA promoter
AR ARA70 is an AR coactivator that has been shown to enhance DHT- and adiol-mediated AR activation in the DU145 PCa cell line transfected with wild-type AR or mutant AR (5). Therefore, we investigated whether ARA70 contributed to adiol-induced PSA promoter activity. Contrary to our expectation, ARA70 inhibited DHT-induced PSA promoter activity by 43% when ARA70 expression plasmid was cotransfected with wild-type AR into PC-3 cells (Fig. 5; column 2 versus column 8). Moreover, ARA70 inhibited adiol-induced PSA promoter activity by 63% (Fig. 5; column 3 versus column 9). In the presence of the mutated AR, ARA70 enhanced DHT- and adiol-induced PSA promoter activity by 155% and 148%, respectively (Fig. 5; columns 5 and 6 versus columns 11 and 12). The observation that ARA70 enhanced equally DHT- and adiol-mediated PSA promoter activity in the presence of mutated AR demonstrates that ARA70 does not account for the greater androgenic action of adiol compared with DHT in LNCaP cells.

To determine if ligand binding to the AR accounts for adiol’s greater androgenic activity in LNCaP cells compared with DHT, we next compared the affinity of adiol and DHT for the mutant AR. DHT bound the mutant AR in LNCaP cells bound with high affinity (Fig. 6A; $K_d = 0.58\, \text{nm}$), whereas adiol bound the mutant AR with one-third of the affinity of DHT (Fig. 6B; $K_d = 1.90\, \text{nm}$). The observation that the affinity of adiol for AR was less than that of DHT demonstrates that affinity to the AR cannot account for the greater androgenic action of adiol compared with DHT in LNCaP cells.

We investigated next other mechanisms through which adiol-induced AR activity. Nuclear hormone receptors are localized generally in the cytoplasm until ligand binding induced nuclear translocation. After we transfected PC-3 cells with wild AR and mutant AR expression plasmid, we treated the cells with $10^{-9}\, \text{M}$ DHT and adiol for 6 h and extracted nuclear protein. The level of nuclear wild and mutant AR protein then was investigated (Fig. 7A). DHT increased the level of nuclear wild AR (4.5-fold of control) more than adiol (1.5-fold of control). When PC-3 cells were transfected with mutant AR, DHT increased the level of nuclear mutant AR 3.3-fold, whereas nuclear mutant AR level was almost the same, irrespective of AR mutation activity in the transfected cells. DHT ($10^{-10}\, \text{M}$) activated the PSA promoter ~50-fold in PC-3 cells transfected with wild-type AR, whereas adiol ($10^{-10}\, \text{M}$)-mediated activation of the PSA promoter was 82% less than that of DHT (Fig. 5; columns 1–3). DHT-mediated induction of the PSA promoter in PC-3 cells transfected with the mutated AR was decreased by 80% compared with that in the PC-3 cells transfected with wild-type AR (Fig. 5; columns 1–6). However, adiol-mediated induction of the PSA promoter activity was the same in PC-3 cells transfected with either the wild-type or mutated AR (Fig. 5; column 5 versus column 6). Moreover, DHT- and adiol-mediated induction of the PSA promoter was similar in the PC-3 cells transfected with the mutant AR. This latter result, combined with the earlier result that adiol induces greater activation of the PSA promoter than DHT in LNCaP cells, indicates that adiol-induced androgenic activity in LNCaP cells is stimulated by some factors, such as AR coactivators, that are not active in PC-3 cells.
Cultured for 12 h in the presence of indicated concentration of DHT or adiol and harvested. Antibody was used for detection of protein. An antibody to wild AR or a mutated AR was transferred to polyvinylidene difluoride membrane, and NH27 anti-AR antibody was used for detection of AR protein. Band density was measured by NIH Image 1.62. The data are presented as mean.

4/H9262 and LNCaP cells. Nuclear proteins were extracted as described in Materials and Methods (data not shown). Growth curve of LNCaP cells by T in vitro shows a biphasic pattern: high concentration of T causes inhibition of LNCaP cell proliferation (Fig. 2A). Because T level in T-treated mice was relatively high compared with the concentration of T promoting proliferation of LNCaP in vitro, it is believed that the tumorigenesis of LNCaP cells was reduced by high T concentration. Concentration of T and adiol in adiol-treated severe combined immunodeficient mice was 0.11 nM and <3.4 nM, respectively (data not shown). Taken together, the observation that adiol stimulated LNCaP growth but not prostate and seminal vesicle growth suggests that adiol has a strong effect on LNCaP cells expressing a mutated AR without conversion to T as opposed to tissues expressing a wild-type AR.

**DISCUSSION**

The major androgenic product secreted from the adrenal gland is DHEA. We have demonstrated recently that the major DHEA metabolite formed in human prostate tissue is adiol (4). Our observation that adiol exists in PCa tissue after hormone therapy, as opposed to the decrease of DHT that occurs, is consistent with the possibility that adiol plays an important role in PCa progression after hormone therapy. This is supported additionally by the observation that adiol activates a mutated AR.

The presence of androgens in prostate tissue after androgen deprivation (1,6-fold). This result almost was coincident with the result of PC-3 with wild AR or mutant AR (Fig. 4 and Fig. 5). We also investigated the level of nuclear AR protein in LNCaP cells treated with DHT and adiol. Treatment of 10^{-10} M adiol for 12 h induced a 3.5-fold nuclear AR protein level increase in LNCaP cells compared with only a 1.5-fold increase induced by equimolar levels of DHT (Fig. 7B). This result suggests that adiol’s ability to induce AR translocation contributes to adiol’s ability to promote a greater androgenic response than DHT.

Because adiol functions as a strong androgen in LNCaP cells in vitro, we investigated whether this activity occurred in vivo. Levels of the T precursors DHEA and adiol are low in rodents (12). Mice are good models to use to manipulate these levels. After male severe combined immunodeficient mice were castrated, they were divided into three groups: no treatment, adiol-treated, and T-treated groups. Then 2 \times 10^6 LNCaP cells were implanted in these mice. Twenty days after inoculation of LNCaP cells, tumors were detected in 0 of 12, 6 of 12, and 1 of 11 of the no treatment, adiol-implanted, and T-implanted mice, respectively (Fig. 8A). At 50 days, tumors were detectable in the no treatment group (3 of 12), and there was no significant difference between the castrated mice and the DHT-treated mice. However, tumors in the adiol-treated mice were significantly larger than those in the other two groups (Fig. 8A). Fifty days after inoculation of LNCaP cells, the mice were killed, and weights of the ventral prostate and seminal vesicle were measured (Fig. 8B). The weights of the prostate and seminal vesicle in the castrated mice were 14.0 and 11.6 mg, respectively, and those in T-treated mice were 169.8 mg and 161.6 mg, respectively. However, adiol implantation induced only a slight increase in the prostate and seminal vesicle weights compared with the castrated group (28.6 mg and 17.4 mg, respectively). We also examined the serum T level in all of the mice and the serum adiol level in the adiol-treated mice. T levels in castrated mice and T-treated mice were <0.1 nM and 31 nM (data not shown). Growth curve of LNCaP cells by T in vitro shows a biphasic pattern: high concentration of T causes inhibition of LNCaP cell proliferation (Fig. 2A). Because T level in T-treated mice was relatively high compared with the concentration of T promoting proliferation of LNCaP in vitro, it is believed that the tumorigenesis of LNCaP cells was reduced by high T concentration. Concentration of T and adiol in adiol-treated severe combined immunodeficient mice was 0.11 nM and <3.4 nM, respectively (data not shown). Taken together, the observation that adiol stimulated LNCaP growth but not prostate and seminal vesicle growth suggests that adiol has a strong effect on LNCaP cells expressing a mutated AR without conversion to T as opposed to tissues expressing a wild-type AR.

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The major androgenic product secreted from the adrenal gland is DHEA. We have demonstrated recently that the major DHEA metabolite formed in human prostate tissue is adiol (4). Our observation that adiol exists in PCa tissue after hormone therapy, as opposed to the decrease of DHT that occurs, is consistent with the possibility that adiol plays an important role in PCa progression after hormone therapy. This is supported additionally by the observation that adiol activates a mutated AR.

The presence of androgens in prostate tissue after androgen deprivation (1,6-fold). This result almost was coincident with the result of PC-3 with wild AR or mutant AR (Fig. 4 and Fig. 5). We also investigated the level of nuclear AR protein in LNCaP cells treated with DHT and adiol. Treatment of 10^{-10} M adiol for 12 h induced a 3.5-fold nuclear AR protein level increase in LNCaP cells compared with only a 1.5-fold increase induced by equimolar levels of DHT (Fig. 7B). This result suggests that adiol’s ability to induce AR translocation contributes to adiol’s ability to promote a greater androgenic response than DHT.

Because adiol functions as a strong androgen in LNCaP cells in vitro, we investigated whether this activity occurred in vivo. Levels of the T precursors DHEA and adiol are low in rodents (12). Mice are good models to use to manipulate these levels. After male severe combined immunodeficient mice were castrated, they were divided into three groups: no treatment, adiol-treated, and T-treated groups. Then 2 \times 10^6 LNCaP cells were implanted in these mice. Twenty days after inoculation of LNCaP cells, tumors were detected in 0 of 12, 6 of 12, and 1 of 11 of the no treatment, adiol-implanted, and T-implanted mice, respectively (Fig. 8A). At 50 days, tumors were detectable in the no treatment group (3 of 12), and there was no significant difference between the castrated mice and the DHT-treated mice. However, tumors in the adiol-treated mice were significantly larger than those in the other two groups (Fig. 8A). Fifty days after inoculation of LNCaP cells, the mice were killed, and weights of the ventral prostate and seminal vesicle were measured (Fig. 8B). The weights of the prostate and seminal vesicle in the castrated mice were 14.0 and 11.6 mg, respectively, and those in T-treated mice were 169.8 mg and 161.6 mg, respectively. However, adiol implantation induced only a slight increase in the prostate and seminal vesicle weights compared with the castrated group (28.6 mg and 17.4 mg, respectively). We also examined the serum T level in all of the mice and the serum adiol level in the adiol-treated mice. T levels in castrated mice and T-treated mice were <0.1 nM and 31 nM (data not shown). Growth curve of LNCaP cells by T in vitro shows a biphasic pattern: high concentration of T causes inhibition of LNCaP cell proliferation (Fig. 2A). Because T level in T-treated mice was relatively high compared with the concentration of T promoting proliferation of LNCaP in vitro, it is believed that the tumorigenesis of LNCaP cells was reduced by high T concentration. Concentration of T and adiol in adiol-treated severe combined immunodeficient mice was 0.11 nM and <3.4 nM, respectively (data not shown). Taken together, the observation that adiol stimulated LNCaP growth but not prostate and seminal vesicle growth suggests that adiol has a strong effect on LNCaP cells expressing a mutated AR without conversion to T as opposed to tissues expressing a wild-type AR.
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vation provides a mechanism through which the AR can be activated after therapy. Our results that DHT levels decrease by 75% are somewhat in conflict with a previous report that demonstrated androgen levels in PCa tissue decrease by only 50–60% after castration (13). Perhaps methodologies account for these differences. Geller et al. (14) described that adrenal androgens DHEA and 4-dione contribute one-fifth to one-sixth of total DHT in prostate tissue and might contribute significantly to the hormone-dependent PCa cells in at least 10% of patients. In the present study, 4-dione and DHEA did not stimulate LNCaP cell proliferation and PSA mRNA expression, and 4-dione had weak androgenic activity to induce PSA promoter in PC-3 cells transfected with wild AR, although these cells might not have enough converting activity from 4-dione to DHT. Once AR in PCa has mutation during hormone therapies, residual adiol, even at low concentration, in prostate tissue also may contribute to relapse of PCa and residual DHT.

The mechanism that maintains adiol levels in PCa tissues after androgen deprivation is not known. Conversion of DHEA to adiol is the only freely reversible reaction in steroid metabolism. To date, five human 17β-hydroxysteroid dehydrogenases (HSDs) have been characterized fully, each showing oxidation or reduction preferences (15). Type 3 17β-HSD converts DHEA to adiol, and type 2 17β-HSD converts adiol to DHEA. Koh et al. (16) demonstrated that the expression levels of the type 3 17β-HSD gene in PCa tissue were significantly higher than in nonmalignant tissues and that expression of type 2 17β-HSD in PCa was significantly lower than in nonmalignant tissues. This result suggests that a part of DHEA in prostate tissue is converted to adiol but that the reverse reaction occurs at a limited level in PCa tissue. This finding may account for the continued presence of adiol in PCa tissue after castration.

Fenton et al. (17) demonstrated that LNCaP-type AR mutant is similar or less sensitive to 4-dione than wild-type AR. Our data are similar to those reported in their article. In contrast, adiol stimulated LNCaP cells proliferation, PSA mRNA expression, and PSA promoter activity at lower concentration. This is the first report of this phenomenon to our knowledge. They also demonstrated that 100 nm bicalutamide blocked the effect of DHT and 4-dione on androgen response element in CV-1 cells transfected with mutant AR (17). In our study, 100 nm bicalutamide inhibited partially adiol’s activity in LNCaP cells. Because adiol has strong androgenic activity even at low concentration in LNCaP cells, higher bicalutamide concentration was necessary to inhibit adiol activity.

We hypothesized that one of mechanisms by which adiol activates mutant AR in LNCaP cells is by enhancing the AR to bind to coactivators. Our results thatARA70 increased PSA promoter activity induced by adiol, albeit slightly, are consistent with the previous reports that describe the AR coactivator ARA70 increased adiol-induced mouse mammary tumor virus promoter activity in DU-145 cells transfected with wild-type AR or mutant AR (5). In the current report, we observed that this induction was not specific to adiol because the same action was found when DHT was used. However, we cannot deny that ARA70 and other coactivators may act cooperatively.

An unexpected finding was that ARA70 reduced adiol-induced PSA promoter activity in the presence of wild-type AR but not mutant AR. This novel finding suggests that a steroid receptor cofactor can act as a coactivator or a corepressor depending on the state (i.e., wild-type versus mutant) of the nuclear hormone receptor.

Because there are many other nonspecific and specific AR coactivators, it is necessary to investigate them and ARA70. AR coactivators should be expressed more in LNCaP cells because adiol-induced androgenic activity in LNCaP cells was much higher than in PC-3 cells. We examined previously the expression level of ARA55, SRC1, ARA54, TIF2, and RAC3 mRNA. However, none of them expressed more in LNCaP cells than in PC-3 cells (18). These data suggest that at least other coactivators may be involved in adiol-induced androgenic activity in LNCaP cells.

Nuclear import of nuclear hormone receptors is mediated through a well-characterized nuclear location signal sequence, NL1, adjacent to the receptor DNA binding domain, and a second uncharacterized motif, NL2, which overlaps with the ligand binding domain (19). Several proteins, such as β-catenin, filamin, importin, Ran, and nuclear transport factor 2, mediate the translocation (20–22). The observation that adiol increased AR import into nuclei more than DHT did in LNCaP cells suggests that adiol facilitates the interaction between mutant AR and these proteins related to nuclear transport.

In conclusion, we identified adiol is present in PCa tissue after androgen deprivation therapy at the same levels of benign prostate hypertrophy tissue without hormone treatment. Furthermore, low concentration of adiol can stimulate proliferation of PCa with mutated AR. Taken together, this provides the opportunity for adiol to promote progression of PCa that has developed a mutated AR during androgen deprivation therapy and 25–40% of DHT in PCa tissue after hormone therapies. Several trials using ketoconazole, which inhibits adrenal androgen synthesis, have been carried out recently for patients with advanced PCa, which progresses after androgen deprivation (23). The effect of inhibition of adrenal androgen synthesis has been recognized. However, a severe toxicity also has been observed because of reduction of other steroids. Our results suggest that in addition to antiandrogens, such as bicalutamide, inhibitors that block selectively synthesis of DHEA and adiol may be useful for extending the period when PCa is sensitive to androgen deprivation therapy without severe side effects.

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The Adrenal Androgen Androstenediol Is Present in Prostate Cancer Tissue after Androgen Deprivation Therapy and Activates Mutated Androgen Receptor

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