Impaired Dihydrotestosterone Catabolism in Human Prostate Cancer: Critical Role of AKR1C2 as a Pre-Receptor Regulator of Androgen Receptor Signaling

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Abstract

We previously reported the selective loss of AKR1C2 and AKR1C1 in prostate cancers compared with their expression in paired benign tissues. We now report that dihydrotestosterone (DHT) levels are significantly greater in prostate cancer tumors compared with their paired benign tissues. Decreased catabolism seems to account for the increased DHT levels when expression of AKR1C2 and SRD5A2 was reduced in these tumors compared with their paired benign tissues. After 4 h of incubation with benign tissue samples, 3H-DHT was predominantly catabolized to the 5α-androstane-3α,17β-diol metabolite. Reduced capacity to metabolize DHT was observed in tumors samples from four of five freshly isolated pairs of tissue samples, which paralleled loss of AKR1C2 and AKR1C1 expression. LAPC-4 cells transiently transfected with AKR1C1 and AKR1C2, but not AKR1C3, were able to significantly inhibit a dose-dependent, DHT-stimulated proliferation, which was associated with a significant reduction in the concentration of DHT remaining in the media. R1881-stimulated proliferation was equivalent in all transfected cells, showing that metabolism of DHT was responsible for the inhibition of proliferation. PC-3 cells overexpressing AKR1C2 and, to a lesser extent, AKR1C1 were able to significantly inhibit DHT-dependent androgen receptor reporter activity, which was abrogated by increasing DHT levels. We speculate that selective loss of AKR1C2 in prostate cancer promotes clonal expansion of tumor cells by enhancement of androgen-dependent clonal proliferation by reducing DHT metabolism. [Cancer Res 2007;67(3):1361–9]

Introduction

Prostate cancer is the second leading cause of cancer-related deaths among men and has a major effect on the health of older Americans (1). Androgens are essential for prostate cancer development, and their elimination and blockade remain the cornerstone of medical management since Huggins first showed symptomatic response and regression of prostate cancer after androgen elimination (2, 3). Epidemiologic studies have also implicated life-long exposure to androgens as a contributing factor for prostate cancer development due to increased cellular proliferation (4, 5). Dihydrotestosterone (DHT) is the key ligand for the androgen receptor (AR) in the prostate and is locally synthesized predominately from circulating testosterone by 5α-steroid reductase type II (SRD5A2; ref. 6). The clinical importance of this pathway was amply shown in a successful chemopreventive trial in which blocking the prostatic conversion of circulating testosterone to DHT by finasteride, a SRD5A2 inhibitor, led to a significant 24.8% reduction of the incidence of prostate cancer in those treated with the inhibitor compared with the control group (7). Thus, in situ hormone synthesis plays an essential role in the intracellular availability of DHT to interact with AR.

Numerous studies have focused on the in situ synthesis of prostatic DHT and the molecular mechanism of AR activation and function (8, 9). However, little emphasis has been placed on the importance of DHT catabolism in the prostate and what role it might play in regulating the intracellular pool of this critical androgen. In the prostate, DHT is predominately metabolized to the weak androgen 5α-androstane-3α,17β-diol (3α-diol) by 3α-hydroxysteroid dehydrogenase (3α-HSD) type III encoded by AKR1C2 (10, 11). We originally identified this protein by its high affinity for bile salts as the human bile acid binder, whereas others purified it as a 3α-HSD or a dihydriodiol dehydrogenase (12–14). Subsequent studies revealed that AKR1C2 is one of four highly related AKR1C subfamily members that have unique substrate specificity and tissue distribution, despite sharing >84% sequence identity (10, 15). In the human prostate, three of these family members are expressed (11), and they have the following enzymatic activities pertinent to androgen metabolism: AKR1C2, 3α-HSD; AKR1C1, 3β-HSD with minor 3α-HSD (97% identity; ref. 16); and AKR1C3, 17β-HSD type V (84% identity). DHT is predominately metabolized to 3α-diol by AKR1C2 and 3β-diol by AKR1C1, whereas AKR1C3 can convert androstendione to testosterone but has little 3α-HSD activity for DHT (10, 17).

We previously observed the selective reduction of AKR1C2 and AKR1C1, but not AKR1C3, gene expression in tumor samples compared with their paired benign tissue (11). Our findings have now been extended to establish that loss of these genes is associated with ~2-fold reduced ability to catabolize 3H-DHT to 3α-diol, its major metabolite, in freshly isolated tumors compared with their paired benign tissues. Furthermore, using prostate cancer cell lines, we have found that increased AKR1C2 or AKR1C1, but not AKR1C3, could inhibit DHT-stimulated growth and AR...
signaling, which was associated with reduced DHT levels in the media. In contrast, no inhibition in proliferation or AR reporter activity was found when using the nonmetabolizable androgen R1881, confirming that catabolism can modify DHT-stimulated proliferation and AR function. Thus, androgen catabolism, in addition to synthesis, can indirectly regulate the activity of AR and thereby provide new therapeutic targets for the treatment of prostate cancer.

Table 1. DHT levels compared with relative expression fold changes of AKR1Cs, SRD5A1, SRD5A2, KRT8, and SM1 in paired human prostate tumor tissues versus prostate benign tissues

<table>
<thead>
<tr>
<th>Sample code</th>
<th>Diagnosis and Gleason grade</th>
<th>Ages</th>
<th>AKR1C2 (mean ± SD)</th>
<th>AKR1C1 (mean ± SD)</th>
<th>SRD5A2 (mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>BM-PDPA/3+4</td>
<td>61</td>
<td>−36.7 ± 2.7</td>
<td>−11.7 ± 1.2</td>
<td>−2.8 ± 0.3</td>
</tr>
<tr>
<td>2</td>
<td>BM-PDPA/3+4</td>
<td>61</td>
<td>−12.9 ± 1.2</td>
<td>2.1 ± 0.4</td>
<td>−12.8 ± 1.2</td>
</tr>
<tr>
<td>3</td>
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<td>−6.3 ± 0.5</td>
</tr>
<tr>
<td>4</td>
<td>BPDA/4+5</td>
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<td>−4.8 ± 0.4</td>
<td>−10.8 ± 0.9</td>
<td>1.2 ± 0.1</td>
</tr>
<tr>
<td>5</td>
<td>PA/4+3</td>
<td>67</td>
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<td>2.8 ± 0.5</td>
<td>−8.2 ± 0.6</td>
</tr>
<tr>
<td>6</td>
<td>PA/3+3</td>
<td>57</td>
<td>−77.8 ± 5.2</td>
<td>−14.3 ± 1.2</td>
<td>ND</td>
</tr>
<tr>
<td>7</td>
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<tr>
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<td>BM-PDPA/3+4</td>
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<td>−21.4 ± 1.9</td>
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<td>−10.2 ± 0.2</td>
</tr>
<tr>
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<tr>
<td>13</td>
<td>BM-PDPA/3+4</td>
<td>58</td>
<td>1.5 ± 0.2</td>
<td>1.8 ± 0.2</td>
<td>1.7 ± 0.1</td>
</tr>
</tbody>
</table>

NOTE: DHT levels in paired human prostate tumor tissues versus prostate benign tissues were measured and compared with relative gene expression fold changes of AKR1Cs, SRD5A1, SRD5A2, KRT8, and SM1. Positive values of gene expression fold changes refer to increased expression in tumors whereas negative values represent decreased expression in tumors compared with paired benign tissues. *P* is the outcome of a paired *t* test with two tails.

Abbreviations: NA, not available in both normal and cancer tissues; ND, not detectable in both normal and cancer tissues; PA, prostatic adenocarcinoma; BMPA, bilateral moderate prostatic adenocarcinoma; BPDA, bilateral poorly differentiated prostatic adenocarcinoma; BM-PDPA, bilateral multifocal to poorly differentiated prostatic adenocarcinoma; PT, prostate tumor; PN, prostate normal tissue; W, weight.

Figure 1. Representative histology of paired tumor and benign appearing prostatic tissue samples. Prostatic tissue slices were processed, stained with H&E, and reviewed to assess tumor stage and homogeneity of resected tissue samples. Histology of two representative pairs of tumor (T) and benign-appearing (N) tissue samples are shown.
Materials and Methods

Prostate tissue processing and hormone measurements. After Institutional Review Board approval, 13 freshly isolated and intact human prostatectomy samples (primary cancers) were immediately cut into 5- or 6-mm slices, and visibly apparent tumors in the posterior zone of the prostate were removed with 6-mm punch biopsy or a 5-mm punch biopsy if tumor was limited. Benign-appearing tissue was isolated from anterior or central zone with a 6-mm punch biopsy. Tumors were staged by standard histology, and the purity of tumor and benign-appearing tissue samples was confirmed by reviewing tissue sectioning punch biopsy sites yielding highly enriched tumor and benign tissue samples minimally contaminated with tumor. Qualified freshly isolated samples were used for RNA isolation, DHT measurement, or incubations with 3H-DHT for monitoring of DHT metabolism.

DHT measurement. Prostate tissue samples were homogenized in 0.1 mol/L phosphate buffer (pH 7.4; assay buffer); internal standard (1H-DHT) was added to follow procedural losses; and the homogenate was centrifuged. Steroids in the supernatant were then extracted with ethyl acetate/hexane (3:2, v/v). After evaporating the organic solvents, the residue was reconstituted in isooctane and applied on a Celite partition chromatography column using ethylene glycol as a stationary phase and toluene in isooctane as the mobile phase. The solvents in the eluted fractions [10% (v/v) toluene in isooctane] containing DHT were evaporated; the residue was reconstituted in assay buffer; and DHT was quantified by a sensitive and specific RIA (18). This method was also used in cellular proliferation studies.

DHT metabolism. Fresh pairs of prostate tumor and benign tissue samples were minced in 2 mL of RPMI 1640 supplemented with 100 μmol/L NADP(H). A total of 0.08 μCi (124 Ci/mmol; final concentration, 320 pmol/L) of 1H-DHT (Perkin-Elmer Life Science, Boston, MA) was added to 2 mL of the incubation media. Tissue and media were extracted following 4 h of incubation in a shaking water bath maintained at 37°C. DHT metabolism was terminated by adding the extraction solvent mixture ethyl acetate/hexane (3:2, v/v), and the residue was reconstituted in methanol. 3H-DHT and its metabolites were well resolved by the reverse-phase high-performance liquid chromatography (HPLC) method of O'Donnell et al. (19) employing a Waters Spherisorb ODS-2 column (5 mm, 250 mm × 4 mm). A Shimadzu LC-10AT HPLC system was used in series with an IN/US JRAM 2B detector to monitor and quantify the radioactivity by continuously mixing 1 mL/min of the effluent with 3 mL/min of ScintiVerse scintillation cocktail (Fisher, Tustin, CA).

Cell culture. The PC-3 cell line was purchased from the American Type Culture Collection (Rockville, MD), and the LAPC-4 cell line was kindly provided by Dr. Sawyers (Department of Medicine and Howard Hughes Medical Institute, University of California at Los Angeles, Los Angeles, CA). Both cell lines were cultured in phenol red-free RPMI 1640 with 10% fetal bovine serum (FBS; Invitrogen, Carlsbad, CA). Permanently transfected PC-3 cell lines expressing AKR1C1, AKR1C2, or AKR1C3 were developed as described (11) and used to assess the effects of AKR1C family members on the DHT- and AR-dependent activation of a mouse mammary tumor virus (MMTV) promoter. For these studies, 4% charcoal/dextran–treated FBS (HyClone, Logan, UT) was used with DHT or R1881 treatments.

Western blot. AKR1C expression was monitored by Western blot with α1850 antiserum that recognizes each AKR1C family member as previously described (11, 20).

Quantitative real-time PCR. Relative expression of AKR1Cs, SRD5A1, and SRD5A2 was done using a gene-specific real-time PCR as described (11, 20), in which their expression in paired tissues were compared with RNase P and reported as mRNA fold change by dividing expression levels in tumor by its paired normal expression level. Primer/probe sequences for real-time PCR are listed in Table S1. Total RNA was isolated using RNaseeasy Midi kit (Qiagen, Valencia, CA) as described (11, 20). cDNA libraries for Taqman quantitative real-time PCR were made using the Omniscript kit (Qiagen) primed with random hexamers (Applied Biosystems, Foster City, CA). Statistical analysis was used to compare gene expression profile, DHT levels, and DHT metabolism as previously described (11, 20, 21).

Cell proliferation assay. Nine micrograms of pSVL-AKR1C1, pCED4-AKR1C2, or pcDNA3.1(+)AKR1C3 encoding the corresponding AKR1C family members in combination with 3 μg of CMV-eGFP-c2 plasmid (kindly provided by Dr. Debbie Johnson, Keck School of Medicine, University of Southern California) were transiently transfected into LAPC-4 cells at 80% to 90% confluence plated in 10-cm dishes as previously described (20). Following transfection, batches of cells were allowed to recover for 24 h and then grown in RPMI 1640 supplemented with 4% charcoal/dextran–treated FBS and 1 nmol/L DHT. Following recovery, a portion of the cells was applied onto a Becton Dickinson FACSCalibur (BD Biosciences, Rockville, MD) to assess transfection efficiency. Pools of transfected cells were then seeded onto six-well plates and maintained in RPMI 1640 supplemented with 4% charcoal/dextran–treated FBS, 3 μL of cell culture medium were changed daily and supplemented with 1 or 10 nmol/L DHT or R1881, and cells were counted for three consecutive days as previously described (20). Real-time PCR was used to document gene expression levels of AKR1C family members 2 days after the transfection.

Effect of AKR1Cs on DHT-dependent AR transactivation. PC-3 cell lines expressing different amounts of AKR1Cs, along with a stable, vehicle–transfected PC-3 cell line used as a control, were transiently transfected with 2 μg pMMTV-Luc (22), 0.2 μg pCMV-hAR expression plasmid (kindly provided by Dr. Gerhard A. Coetsee, Keck School of Medicine, University of California at Los Angeles, Los Angeles, CA).

Table 1. DHT levels compared with relative expression fold changes of AKR1Cs, SRD5A1, SRD5A2, KRT8, and SM1 in paired human prostate tumor tissues versus prostate benign tissues (Cont’d)

<table>
<thead>
<tr>
<th>SRD5A1 (mean ± SD)</th>
<th>KRT8 (mean ± SD)</th>
<th>SM1 (mean ± SD)</th>
<th>DHT in PT (ng/g W)</th>
<th>DHT in PN (ng/g W)</th>
<th>DHT PT/PN fold changes</th>
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</thead>
<tbody>
<tr>
<td>1.3 ± 0.1</td>
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<td>−1.1 ± 0.1</td>
<td>11.3</td>
<td>7.24</td>
<td>1.56</td>
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<td>−1.7 ± 0.1</td>
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<td>5.32</td>
<td>1.57</td>
</tr>
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<td>−2.6 ± 0.2</td>
<td>−1.1 ± 0.2</td>
<td>1.7 ± 0.1</td>
<td>3.68</td>
<td>2.35</td>
<td>1.57</td>
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<tr>
<td>1.4 ± 0.1</td>
<td>1.4 ± 0.4</td>
<td>1.2 ± 0.3</td>
<td>6.10</td>
<td>5.91</td>
<td>1.03</td>
</tr>
<tr>
<td>2.9 ± 0.3</td>
<td>−1.1 ± 0.3</td>
<td>−1.9 ± 0.1</td>
<td>10.7</td>
<td>6.15</td>
<td>1.74</td>
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<td>−1.9 ± 0.1</td>
<td>1.4 ± 0.1</td>
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<td>6.59</td>
<td>4.88</td>
<td>1.35</td>
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<tr>
<td>ND</td>
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<td>1.72</td>
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<td>1.10</td>
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<tr>
<td>−3.1 ± 0.3</td>
<td>−1.2 ± 0.2</td>
<td>1.3 ± 0.1</td>
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<tr>
<td>1.8 ± 0.2</td>
<td>NA</td>
<td>NA</td>
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<tr>
<td>1.3 ± 0.1</td>
<td>1.3 ± 0.2</td>
<td>1.5 ± 0.6</td>
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</table>

P = 0.012

1.42 ± 0.25 (mean ± SD)
Luminoskan Ascent Machine (Labsystems, Franklin, MA) with the Dual Luciferase Activity kit (Promega). The ratio of firefly to Renilla luciferase activity was normalized to total protein to compare promoter activity from different cell lines. Luciferase activity of control PC-3 cells transfected with vehicle vector was arbitrarily defined as 100 ± SD units. Experiments were repeated three times in triplicate.

Results

DHT levels in paired prostate cancer tumor and benign tissue samples. Little is known about the cellular localization of AKR1C1 and AKR1C2 proteins in the prostate as their high amino acid sequence homology (97% identity) prevents differentiating each other from AKR1C3, which shares 86% sequence identity. We have previously developed an antiserum that cross-reacts with AKR1C1 and AKR1C3 but not AKR1C2 (20). In contrast, AKR1C3-specific polyclonal antisera and a monoclonal antibody are available as its sequence significantly diverges from the other family members at its COOH-terminal end. In normal prostate tissue, Fung et al. immunolocalized AKR1C3 using a monoclonal antibody on endothelial, perineural, smooth muscle cells, and stromal cells with minimal staining in the epithelium (23, 24). In contrast, El-Alfy et al. localized AKR1C3 predominately on the basal epithelium and luminal cells using a polyclonal AKR1C3-specific antiserum, which was confirmed by in situ hybridization (25, 26). Another report using the same antiserum also showed a similar immunohistochemical staining pattern (27). To identify which populations of prostatic cells expressed AKR1C1 and AKR1C2, transcriptome analysis of four highly enriched populations of luminal, basal, stromal, and endothelial cells from the prostate was queried using the SCGAP Urologic Epithelial Stem Cells Project web site5 (28). Relative expression profile from the most abundant to least revealed the following expression patterns: AKR1C1, stromal, luminal > endothelial >> basal; AKR1C2, stromal, luminal > endothelial > basal; and AKR1C3, basal, endothelial > stromal > luminal cells. These studies indicate that AKR1C1 and AKR1C2 transcripts are equivalently expressed in the stromal and acinar components. Highly enriched tumors with corresponding paired benign tissue samples were harvested and confirmed by histologic evaluation, as illustrated in Fig. 1. Table 1 lists the pathologic diagnosis, DHT levels, and the relative changes in gene expression responsible for either DHT catabolism (AKR1C2 and AKR1C1) or DHT synthesis (SRD5A1 and SRD5A2) in these paired prostate samples, compared with RNAse P, whose expression is unchanged in prostate cancer (11). Relative enrichment of stromal or epithelial content for each sample was broadly assessed by comparing the relative expression profile of the human keratin 8 gene (KRT8), a recognized marker for the epithelial compartment, and the human myosin heavy polypeptide 11, smooth muscle (SM1), a stromal-specific marker (29, 30). In these paired samples, relative expression of stromal or epithelial markers was closely matched. Approximately three quarters of the patients had a relative loss of AKR1C2 and/or AKR1C1 in their tumor samples. As AKR1C1 and AKR1C2 are equivalent expressed in both stroma and epithelium, relative enrichment of either component in tumor samples could not account for the observed loss of AKR1C1 or AKR1C2 gene expression. These findings are in close agreement with our prior findings (11). Relative reduction of SRD5A2 expression (>4-fold), but not SRD5A1, in prostate tumors were found in 5 of 13 paired samples, consistent with previous studies (29). The average DHT levels detected in our paired prostate tissues were comparable with previously reported values. When changes in DHT levels were compared within individual pairs, a 42% higher DHT levels was detected on average in prostate tumors compared with their paired benign tissue samples ($P < 0.05$). As SRD5A2 expression was decreased, we reasoned that increased DHT synthesis was unlikely to account for the greater DHT levels in these samples.

Reduced DHT metabolism in prostate tumor tissues compared with paired benign tissues. We developed in vitro incubations of freshly harvested prostatic tissues to assess if loss of AKR1 Cs was associated with altered metabolism of exogenously supplied $^3$H-DHT by using a radio-inverse phase HPLC (11, 19). To assess the importance of AKR1Cs in the metabolism of DHT in benign tissue, the non–substrate-competitive inhibitors indomethacin (100 μmol/L) or tolmetin (200 μmol/L) were added to the media. We (31) and Steckelbroeck et al. (16) have previously used these agents to inhibit the enzymatic activity of AKR1Cs within cells to determine their relative contribution to either androgen metabolism or intracellular distribution and transport of bile salts by primary hepatocytes. In two independent benign tissue samples, indomethacin inhibited the loss of $^3$H-DHT by ~3-fold in each case (data not shown). In one sample, the AKR1C inhibitor tolmetin was also as effective at inhibiting the loss of $^3$H-DHT, thereby confirming that the AKR1Cs are required for the rapid catabolism of $^3$H-DHT.

DHT metabolism in five pairs of prostate tissue samples was then quantified using this radio-HPLC, and results were compared with their relative AKR1C1 and AKR1C2 gene expression. The $^3$H-DHT data were normalized to tissue weight to allow cross-comparisons of changes in the relative radioactivities of $^3$H-DHT and its metabolites with those of the endogenous DHT levels. Within individual pairs, results from tumors were compared with their corresponding benign tissues, which were assigned a value of 100. As shown in Fig. 2A, greater $^3$H-DHT retention was found in four of the five prostate tumors compared with their paired benign tissues, and as a group, significantly greater $^3$H-DHT retention was found in these tumors compared with their paired benign tissues ($P < 0.05$). As the average relative retention of $^3$H-DHT was 2-fold greater in tumors compared with their corresponding benign tissues ($P < 0.05$), isotope dilution within their respective endogenous intracellular pool of DHT (Table 1) could not account for this significantly greater retention of $^3$H-DHT. Moreover, as shown in Fig. 2B, the radioactivity associated with the 3α-diol metabolite in the tumors consistently accounted for a lower fraction (54 ± 15%) of the combined radioactivity present in the elution of DHT and its metabolites compared with benign tissues ($P < 0.01$). As shown in Fig. 2C, production of the 3β-diol metabolite was also reduced in tumors, and the 3β-diol metabolite only constituted ~20% of all the DHT metabolites present in the incubation from benign tissues. In Fig. 2D, relative changes in AKR1C2 and AKR1C1 expression in tumors compared with paired benign tissues paralleled their reduction in DHT metabolism. No correlation was found between DHT metabolism and the expression

5 http://scgap.systemsbiology.net/.
profile of SRD5A1 and SRD5A2 (data not shown). Three other pairs of samples processed using TLC chromatography also showed a similar retention of $^3$H-DHT in tumor samples relative to their paired benign tissues (data not shown). Taken together, these results indicate that metabolism of DHT to 3α-diol was significantly impaired in tumors compared with their paired benign tissues.

Inhibition of DHT-dependent growth of LAPC-4 cells by expression of AKR1C1 and AKR1C2. Similar to the prostate cancer cell lines PC-3, DU-145, and LNCaP (11), LAPC-4 cells minimally expresses AKR1C1 and AKR1C2, in contrast to AKR1C3 (data not shown). LAPC-4 cells were chosen for these studies because they express a wild-type AR, in contrast to the AR expressed in LNCaP cells that harbors a mutation in the ligand-binding domain, which permits binding by other class of steroids (32). LAPC-4 cells were individually transfected with AKR1C family members to determine if they can modify androgen-stimulated cellular proliferation. Transfection efficiencies for AKR1C1, AKR1C2, and AKR1C3 expression plasmids in LAPC-4 cells were, correspondingly, 43%, 44%, and 38% for these studies. LAPC-4 cells were transiently transfected with AKR1C1, AKR1C2, or AKR1C3 expression plasmids, which resulted in >5,000-fold increased in AKR1C gene expression, and their growth in response to 1 or 10 nmol/L DHT was compared with LAPC-4 cells transfected with comparable amount of empty vector. In Fig. 3, transfection with pSVL-AKR1C1 (A) or pCED4-AKR1C2 (B) significantly reduced dose-dependent DHT-stimulated proliferation compared with vehicle vectors. However, no inhibition was observed when the nonmetabolizable androgen R1881 was used to stimulate cellular proliferation. In addition, proliferations of nontransfected LAPC-4 cells by R1881 treatments were comparable with LAPC-4 cells transfected with vehicle vector or expression plasmids, which were also observed with DHT treatments (data not shown). AKR1C2 was more effective at inhibiting DHT-stimulated proliferation compared with AKR1C1-transfected LAPC-4 cells. Transfection with AKR1C2 significantly suppressed 1 and 10 nmol/L DHT-stimulated cellular proliferation ($P < 0.01$), whereas AKR1C1-transfected cells were only able to significantly reduce 1 nmol/L DHT-dependent proliferation ($P < 0.05$). In contrast, as shown in Fig. 3C, cells transfected with pcDNA3.1(+)AKR1C3 showed no inhibition of DHT- or R1881-stimulated growth. As shown in Fig. 3D, DHT levels in LAPC-4 cells transfected with AKR1C1 and AKR1C2 were significantly reduced to, respectively, ~80% and 30% of that remaining in the media of LAPC-4 cells transfected with AKR1C3 or vector controls. As R1881-stimulated proliferation was not reduced by AKR1C1 and AKR1C2, our findings confirm that inhibition of DHT-dependent proliferation was not due to metabolism of another substrate or some other unknown function of AKR1C1 or AKR1C2, such as inhibition of the cell cycle. The significant reduction in DHT levels after 4 h implicates reduction in

Figure 2. DHT metabolism in short-term incubations is impaired in prostate cancer. A, relative amounts of $^3$H-DHT retained in five individual pairs of benign and prostate cancer samples after 4 h of incubation. $^3$H-DHT radioactivity quantified by radio-HPLC was initially expressed per gram of tissue, which was then normalized for each pair as the ratio of values in tumors divided by paired benign tissue sample, which was designated as 100. Significantly higher ratio 2.0 ± 0.7 (mean ± SD; $P < 0.05$) of relative $^3$H-DHT levels were retained in the combined prostate tumors compared with their paired benign tissue samples. B, relative $^3$H-3α-diol values in tumors were compared with their corresponding paired benign tissue samples, which were normalized to 100. In all tumor samples, $^3$H-3α-diol values contributed a significantly ($P < 0.01$) lower percentage (54 ± 15%) of the combined radioactivity of $^3$H-DHT and its metabolites compared with their paired benign tissue samples, showing a relative reduction in DHT metabolism by tumors. C, relative $^3$H-3β-diol values in tumors were compared with their corresponding paired benign tissue samples, which were normalized to 100. In two of the samples, $^3$H-3β-diol was below levels of detection. The amount of DHT metabolized to $^3$H-3β-diol was significantly ($P < 0.05$) lower in the combined tumor samples compared with their paired benign tissue samples. D, changes in gene expression of AKR1C2 and AKR1C1 in tumors were divided by their corresponding relative expression in paired benign tissue samples and defined as mRNA fold change. Negative number represents a decreased expression in tumor compared with paired benign tissue sample. Bars, SD.
DHT levels as the major cause for reduce proliferation of all LAPC-4 cells present in the wells transfected with AKR1C1 or AKR1C2 plasmids.

Inhibition of DHT-dependent AR activation by AKR1C2. To assess if AKR1C2 could directly block DHT-dependent activation of an AR-driven reporter, PC-3 cells stably expressing AKR1C1, AKR1C2, or AKR1C3 were transfected with a MMTV promoter that harbors a hormone-responsive element along with an AR expression plasmid (33). No luciferase activity was detectable in the absence of AR or DHT (data not shown). In Fig. 4A, AKR1C2 significantly reduced AR- and DHT-dependent MMTV reporter activity (P < 0.01) to a greater extent than AKR1C1, although more AKR1C1 was expressed than AKR1C2. Note that no change in luciferase activity was found in cells overexpressing AKR1C3, confirming that DHT is a poor substrate for this family member. As shown in Fig. 4B, increasing AKR1C2 significantly reduced AR-dependent MMTV reporter activity in a dose response fashion with a single DHT concentration at the P < 0.01 level but had no effect on R1881-dependent activation. Conversely, increasing concentrations of DHT were able to overcome the inhibition of the reporter activation in PC-3 cells permanently expressing the lowest level of AKR1C2 in Fig. 4C. Taken together, these data show that AKR1C2 can influence DHT-dependent gene expression and signaling by promoting DHT metabolism.

Discussion

Prostate cancer and tissue DHT. In prostate cancer cell lines, androgens activate survival pathways that block cell death mediated by either tumor necrosis factor-α or Fas activation.
(34), prevent etoposide-induced cell death (35), inhibit phosphatase and tensin homologue deleted on chromosome 10–induced apoptosis (36), and abrogate the toxicity of FKHR (37). Androgen ablation, by both decreased testosterone synthesis and blockade of AR, effectively reduces AR signaling with both shrinkage of tumors and symptomatic relief. Ultimately, a so-called androgen-independent state evolves in which prostate cancer becomes progressively less responsive to androgen ablation (38). Numerous pathways participate in this process, including AR activation in the absence of ligand and nongenomic effects of AR (38).

Recent studies have implicated increased AR expression as a cause for androgen independence with increased sensitivity to low levels of androgens (9). In cell lines, expression of AR by itself is sufficient to induce androgen-independent features, and selective deletion of AR can reverse tumorigenicity of androgen-independent cells (9, 39).

Prior epidemiologic and pathologic studies have carefully sought for correlations between androgen levels in either serum or within prostate cancer tumors and the risk of development or progression of prostate cancer (4, 40, 41). Mohler et al. reported changes in prostatic androgen levels in primary prostate cancer compared with recurrent disease as well as differences in prostatic androgens among different ethnic groups (40, 41). However, prior studies have failed to directly compare androgen levels in paired tissues, and none have correlated these changes in DHT levels with gene expression of AKR1C2. Most studies have compared production of DHT from testosterone, whereas use of 3H-DHT in our experimental design allowed us to bypass the synthetic pathway to specifically focus on the ability of these samples to catabolize DHT. Kreig et al. also noted significantly greater DHT levels and reduced 3α-diol levels in prostate cancer compared with nonpaired benign tissues (42).

In our present studies, DHT levels were found on average to be 42% higher in tumors compared with paired benign tissues. Over prolonged periods of time, a proportional 42% increase in DHT binding to AR could potentially cause cumulative proliferation and growth of tumors. This would be consistent with the molecular epidemiologic studies of Makridakis et al., who have reported increases in prostate cancer risk associated with functional polymorphism of SRD5A2 that increase catalytic activity (43).

**Role of AKR1C2 as a pre-receptor regulator of AR signaling.**

Steady-state levels of intracellular DHT are maintained through a balance between their local synthetic and catabolic rates. In LAPC-4 cells, metabolism of DHT by AKR1C1 and AKR1C2 was sufficient to inhibit DHT-stimulated proliferation, although a majority of cells were not transfected. However, the significant reduction in DHT levels in the media for all cells exposed to the AKR1C1 or AKR1C2 expression plasmid is likely to have also reduced the DHT-dependent proliferation of the nontransfected cells. In addition, no inhibition of reporter activity was observed when R1881 was used.

**Figure 4.** Effects of AKR1Cs on DHT-dependent activation of AR-dependent MMTV promoter. DHT- and AR-dependent reporter activity was determined with PC-3 cell lines stably expressing AKR1C1, AKR1C2, or AKR1C3 and were compared with cells stably transfected with vehicle vector. A, expression of AKR1C2 was able to significantly reduce AR-dependent MMTV reporter activity by DHT (P < 0.01). AKR1C1 was also able to reduce AR-dependent MMTV reporter activity (P < 0.05) but not to the same extent as AKR1C2. AKR1C3 was unable to reduce DHT-dependent activation of the MMTV promoter. B, increased expression of AKR1C2 in PC-3 cells was able to significantly inhibit AR-dependent MMTV reporter activity with 100 pmol/L DHT (P < 0.01) compared with vehicle vector control. No inhibition of reporter activity was observed when R1881 was used. C, in PC-3 cells expressing the least amount of AKR1C2, increasing concentrations of DHT were able to significantly overcome the inhibition of AR-dependent reporter activation.

**Figure 5.** Proposed model for increased AR signaling due to loss of AKR1C2 and AKR1C1 in prostate cancer. Circulating testosterone (T) diffuses into prostatic cells and is reduced by SRD5A2 to form DHT, the major ligand for the AR. AKR1C2 is responsible for the majority of DHT catabolism and predominately reduces DHT to the weak androgen 3α-diol. AKR1C1 also catalyzes the stereospecific reduction of DHT to the weak androgen 3β-diol. 3α-diol is a ligand for ERα, which promotes antiproliferative response in the prostate. In paired prostate cancer samples, decreased expression of AKR1C2, AKR1C1, and SRD5A2 was observed with increased DHT levels. Our model predicts that reduced AKR1C2 expression in prostate cancer leads to reduced DHT catabolism, resulting in a greater retention of DHT, thereby enhancing AR-dependent DHT signaling in prostate cancer.
inhibition of proliferation was observed with R1881 treatment. Data from promoter activity assay revealed that increasing AKR1C2 expression reduced the AR-DHT driven MMTV promoter activity in PC-3 cell line. In our paired tissue samples, reduced metabolism of DHT was found to correspond with the loss of AKR1C2 and AKR1C1 expression. As SRD5A2 expression was not increased in these prostate cancer samples, the loss of DHT metabolism associated with reduced AKR1C2 expression is probably the major mechanism for the observed increase in DHT levels in the tumor as a result of enhanced retention. Of note, Bauman et al. recently reported comparable levels of AKR1C2 transcripts in epithelial cell lines established from normal and prostate cancer tissue samples (44). Potential explanations for these discrepancies include comparison of whole tissue expression levels with epithelial cell lines established from tissue samples and variation in gene expression profile in these established cell lines due to culture conditions or lack of contact with other cell types.

Currently, androgen ablation is the major treatment for hormone-sensitive prostate cancer and is achieved by inhibition of testicular testosterone synthesis with gonadotropin-releasing hormone receptor antagonists in combination with an AR ligand antagonist (8). However, androgens synthesized from adrenal precursors are only reduced by 50% with these treatments, and Mizokami et al. have reported elevated androst-5-ene-3,17β-diol levels after androgen ablation (45). This and other adrenal derived androgens may be important contributors to AR-dependent gene expression (46). Indeed, Nishiyama et al. reported that prostatic DHT levels remained at 25% of pretreatment levels in patients undergoing androgen deprivation therapy, in sharp contrast to serum levels of testosterone that were decreased by 93% (47). Based on the reasons mentioned above, in situ DHT catabolism mediated by AKR1C2 in the prostate could be developed as a potential adjuvant therapy to androgen ablation for hormone-sensitive prostate cancer by induction of AKR1C2 expression.

We speculate that the selective loss of AKR1C2 in prostate cancer promotes clonal expansion of tumor cells by enhancement of androgen-dependent cellular proliferation. Isaacs et al. have emphasized the acquired remodeling of terminal epithelium from being normally dependent on paracrine stimulation for growth to being independent of these factors after malignant transformation (8, 49). From these studies, we conclude that increased DHT as a result of reduced DHT catabolism in prostate cancer would lead to cellular proliferation in an AR-dependent fashion. Our cellular proliferation experiments showed that increased AKR1C2 expression can reduce DHT-stimulated cell growth, thereby confirming that increased metabolism of DHT can block the activation of AR.

In summary, catabolism of androgens by AKR1Cs can function as effective pre-receptor regulators of AR-dependent gene expression by modulation of the essential ligand for AR. Figure 5 illustrates our model for how reduction of AKR1C2 and possibly AKR1C1 metabolism of DHT in prostate cancer could promote AR signaling by increasing the intracellular DHT levels. According to Steckelbroeck et al., AKR1C2 favors the reduction of DHT to the 3α-diol compared with the 3β-diol metabolites by a ratio of 20:1, whereas AKR1C1 ratio of metabolites is 1:4. In addition, AKR1C2 apparent Kcat activity for 3α-diol production is 2.81 compared with AKR1C1's Kcat of 0.61 for 3β-diol production, which agrees with our findings in Figs. 3 and 4 (16). Thus, AKR1C2 is responsible for reducing DHT to 3α-diol because of its catalytic efficiency and is the major enzyme responsible for DHT catabolism. Although formation of 3β-diol from DHT was relatively minor compared with 3α-diol, 3β-diol is a recognized ligand for estrogen receptor β (ER-β), which promotes antiproliferative pathways (50). Loss of AKR1C1 resulting in decreased production of a potent ER-β ligand would also provide a selective growth advantage for these tumor cells. As SRD5A2 expression is decreased in prostate cancer, we predict that a significant impairment in the catabolic pathway is responsible for the increased DHT levels that we observed in tumor samples. We speculate that selective loss of AKR1C2 and AKR1C1 in prostate cancer promotes clonal expansion of tumor cells by enhancement of androgen-dependent cellular proliferation.

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