Increased expression of type 2 3 α -hydroxysteroid dehydrogenase/type 5 17 β -hydroxysteroid dehydrogenase (AKR1C3) and its relationship with androgen receptor in prostate carcinoma

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Abstract

Type 2 3 α -hydroxysteroid dehydrogenase (3 α -HSD) is a multi-functional enzyme that possesses 3 α -, 17 β - and 20 α -HSD, as well as prostaglandin (PG) F synthase activities and catalyzes androgen, estrogen, progestin and PG metabolism. Type 2 3 α -HSD was cloned from human prostate, is a member of the aldo-keto reductase (AKR) superfamily and was named AKR1C3. In androgen target tissues such as the prostate, AKR1C3 catalyzes the conversion of Δ^4 -androstene-3,17-dione to testosterone, 5 α -dihydrotestosterone to 5 α -androstane-3 α ,17 β -diol (3 α -diol), and 3 α -diol to androsterone. Thus AKR1C3 may regulate the balance of androgens and hence *trans*-activation of the androgen receptor in these tissues. Tissue distribution studies indicate that AKR1C3 transcripts are highly expressed in human prostate. To measure AKR1C3 protein expression and its distribution in the prostate, we raised a monoclonal antibody specifically recognizing AKR1C3. This antibody allowed us to distinguish AKR1C3 from other AKR1C family members in human tissues. Immunoblot analysis showed that this monoclonal antibody binds to one species of protein in primary cultures of prostate epithelial cells and in LNCaP prostate cancer cells. Immunohistochemistry with this antibody on human prostate detected strong nuclear immunoreactivity in normal stromal and smooth muscle cells, perineurial cells, urothelial (transitional) cells, and endothelial cells. Normal prostate epithelial cells were only faintly immunoreactive or negative. Positive immunoreactivity was demonstrated in primary prostatic adenocarcinoma in 9 of 11 cases. Variable increases in immunoreactivity for AKR1C3 was also demonstrated in non-neoplastic changes in the prostate including chronic inflammation, atrophy and urothelial (transitional) cell metaplasia. We conclude that elevated expression of AKR1C3 is highly associated with prostate carcinoma. Although the biological significance of elevated AKR1C3 in prostatic carcinoma is uncertain, AKR1C3 may be responsible for the trophic effects of androgens and/or PGs on prostatic epithelial cells.

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Introduction

Androgens are critical for maintaining normal function of the prostate gland and are also trophic factors for the development of human prostate carcinoma (CaP) (Isaacs *et al.* 1994). The orchestrated expression of androgen-metabolizing enzymes including 5 α -reductase type 2, 17 β -hydroxysteroid dehydrogenase (HSD), 3 α -HSD and 3 β -HSD is critical to maintain the balance of different androgen metabolites within the prostate to maintain its normal physiological function. The type 2 3 α -HSD was cloned from a human prostate cDNA library by us (Lin *et al.* 1997), was found to belong to the aldo-keto reductase (AKR) superfamily, and was designated AKR1C3. AKR1C3 possesses 3 α -HSD, 17 β -HSD, 20 α -HSD and prostaglandin (PG) F synthase activities and catalyzes androgen, estrogen, progesterone and PG metabolism (Dufort *et al.* 1996, Lin *et al.* 1997, Matsuura *et al.* 1998, Penning *et al.* 2001). AKR1C3, therefore, has the capability of regulating ligand access to multiple nuclear receptors including the androgen receptor (AR), estrogen receptor, progesterone receptor and peroxisome proliferator activated receptor gamma (PPAR γ) in endocrine peripheral tissues.

In androgen metabolism, AKR1C3 catalyzes the reductive reaction of 5 α -dihydrotestosterone (5 α -DHT) to 5 α -androstane-3 α ,17 β -diol (3 α -diol) by its 3 α -HSD activity (Lin *et al.* 1997) and the conversion of Δ^4 -androstene-3,17-dione (Δ^4 -dione) to testosterone by its 17 β -HSD activity (Labrie *et al.* 2000a) (Fig. 1). 5 α -DHT and testosterone are active androgens in the prostate, and responsible for physiological and pathological development of the gland. It has been assumed that 3 α -diol has no androgenic effects unless it is first oxidized back to 5 α -DHT (Jacobi *et al.* 1978, Mahendroo *et al.* 1996, Wilson *et al.* 2003a,b). We recently reported that 3 α -diol may stimulate prostate cell proliferation through an AR-independent, epidermal growth factor-like pathway(s) (Nunlist *et al.* 2004, Zimmerman *et al.* 2004). These results would be consistent with previous reports describing 3 α -diol-induced prostate enlargement in a castrated dog model (Jacobi *et al.* 1978), 3 α -diol-regulated male urogenital tract development in the fetus of the pouch of female marsupials (Wilson *et al.* 2003b), and 3 α -diol-induced parturition in a mouse model (Mahendroo *et al.* 1996). AKR1C3, therefore, can be a candidate enzyme in regulating the activation of AR or growth factor signaling pathways through local accumulation of testosterone, 5 α -DHT, or 3 α -diol for prostate growth.

In PG metabolism, AKR1C3 catalyzes the 11-keto-reduction of PGD₂ to yield the PGF_{2 α} epimer,

9 α ,11 β -PGF_{2 α} , thereby depriving PPAR γ of its putative ligand 15 Δ -PGJ₂ (Desmond *et al.* 2003). In normal prostate, epithelial cells do not express PPAR γ but in PC-3 prostate cancer cells, which express PPAR γ , 15 Δ -PGJ₂ is growth inhibitory (Subbarayan *et al.* 2004). 15 Δ -PGJ₂ is derived from normal stromal cells and suppresses adjacent tumor growth (Kim *et al.* 2005). These growth inhibitory properties may be mediated by downregulation of cyclooxygenase (COX)-2 by 15 Δ -PGJ₂ (Sabichi *et al.* 2004). *In vivo*, results with COX-2 inhibitors also show a pronounced inhibitory effect on tumor microvessel density and angiogenesis (Kirschenbaum *et al.* 2001). In the presence of elevated AKR1C3, PGD₂ will be diverted to its pro-proliferative ligand 9 α ,11 β -PGF₂. Thus AKR1C3 may also be intimately involved in controlling proliferative growth and vascular development in the diseased prostate.

Tissue and prostate distribution of AKR1C3 has been investigated. Using an AKR1C3 3'-UTR as a cDNA probe, transcript expression of AKR1C family members including AKR1C2/AKR1C3 was detected in multiple tissues including prostate (Lin *et al.* 1997). Expression of these transcripts was also detected in primary cultures of both prostate epithelial and stromal cells with higher levels of transcript expression in epithelial cells (Lin *et al.* 1997). Semi-quantitative AKR1C isoform-specific RT-PCR confirmed the high expression of AKR1C3 in the prostate (Penning *et al.* 2000). AKR1C3 has been shown to be expressed in the Leydig cells of the testis as well as prostate basal and luminal epithelial cells using an anti-AKR1C3 polyclonal antibody raised against the C-terminal domain of the protein (Pelletier *et al.* 1999). Immunoelectron microscopy shows no association with specific organelles compatible with the cytosolic localization of AKRs (Pelletier *et al.* 2001) but some nuclear staining was noted.

To further study AKR1C3 protein expression in the prostate, we developed and characterized a mouse anti-human 3 α -HSD monoclonal antibody (mAb; clone NP6G6.A6). This mAb is monospecific against AKR1C3 (Lin *et al.* 2004) and does not cross-react with AKR1C1, AKR1C2 or AKR1C4. In this report, we extend our investigation of AKR1C3 distribution in CaP tissues. High levels of immunoreactivity were observed in all endothelial cells in the prostate with staining in some stromal cells. In contrast to low immunoreactivity in normal prostate epithelium, strong positive staining was detected in the epithelium of the majority of CaP cases along with other prostate pathologies. Elevated AKR1C3 expression in human CaP may result in an imbalance of androgen

Figure 1 Summary of AKR1C3-mediated nuclear receptor ligand metabolism. AKR1C3 has 3 α - and 17 β -HSD, and 11-ketoprostaglandin reductase activities.

metabolism in the prostate, which may lead to abnormal prostate growth through de-regulated *trans*-activation of nuclear receptors (AR and PPAR γ).

Materials and methods

Reagents

Mouse anti-human AR mAb was acquired from Vector Laboratories (Burlingame, CA, USA). Mouse anti-human type 2 3 α -HSD (AKR1C3) mAb was prepared at the Cell Center, University of Pennsylvania

School of Medicine as previously described using the full-length recombinant AKR1C3 protein as the antigen (Lin *et al.* 2004). Kenneth's HY, RPMI 1640, keratinocyte serum-free medium (K-SFM), and fetal bovine serum (FBS) were purchased from Invitrogen (Carlsbad, CA, USA), and ORIGEN hybridoma cloning factor was obtained from IGN International (Gaithersburg, MD, USA). Charcoal/dextran-treated FBS was purchased from HyClone (Logan, UT, USA). Hematoxylin and eosin were purchased from Sigma (St Louis, MO, USA). Biotinylated goat anti-mouse

secondary antibody, horseradish peroxidase (HRP)-conjugated streptavidin, and diaminobenzidine tetrahydrochloride (DAB)-H₂O₂ substrate were obtained from BioGenex (San Ramon, CA, USA).

Cell Culture

Human prostate LNCaP cells were obtained from ATCC and maintained in growth medium consisting of RPMI 1640 medium supplemented with 10% FBS, 100 units/ml penicillin and 100 µg/ml streptomycin (Invitrogen) in a humidified cell incubator at 37°C in 5% CO₂. Primary cultures of prostate cells were prepared from prostate following surgical radical prostatectomy as described by Lang *et al.* (2001). Primary cultures of prostate cells were established and maintained in K-SFM supplemented with 1% charcoal/dextran-treated FBS.

The hybridoma line that produces anti-human AKR1C3 mAb was maintained in hybridoma growth media consisting of Kenneth's HY, 20% FBS, 2% hybridoma cloning factor, 4 mM L-glutamine and 1% OPI Media Supplement (Sigma).

Western blot analysis

Cellular proteins were prepared from LNCaP cells and from primary cultures of prostate epithelial cells in a modified RIPA buffer (50 mM Tris-HCl (pH 7.4), 1% NP-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA and 2 mM phenylmethylsulfonyl fluoride). Total soluble proteins (30 µg) were electrophoresed on a 12% Tris-HCl gel (Bio-Rad). Proteins were transferred onto polyvinylidene difluoride (PVDF) membranes (Bio-Rad). The membranes were blocked with 5% non-fat dry milk (Bio-Rad) in Tris-buffered saline containing 1% Tween 20. Antigens were detected by incubating with mouse anti-human AKR1C3 mAb (1 : 500) or anti-β-actin mAb (1 : 5000; Sigma) at room temperature for 2 h followed by incubation with HRP-conjugated anti-mouse IgG (1 : 125 000; KPL, Gaithersburg, MD, USA) secondary antibody at room temperature for another 1 h. Immunoreactive protein was then detected using enhanced chemiluminescence reagent (Pierce, Rockford, IL, USA) according to the manufacturer's protocol.

RT-PCR analysis of AKR1C3 mRNA

Amplification of AKR1C3 and β-actin mRNA was performed using AKR1C3 isoform-specific primers (Penning *et al.* 2000) from total RNA isolated from LNCaP cells and primary cultures of prostate epithelial cells.

Table 1 Summary of age, Gleason score and percentage of AKR1C3-positive cancer cells

Case No.	Age	Gleason score	AKR1C3-positive cancer cells (%)
1	68	4+3	>75
2	69	3+4	>75
3	56	3+4	>75
4	68	3+3	>75
5	59	3+3	50
6	64	3+4	25
7	59	3+4	25
8	60	4+3	25
9	56	3+3	10
10	65	3+4	0
11	52	3+4	0

Human prostate tissues

Archival formalin-fixed, paraffin-embedded prostate tissue blocks and their associated clinical information were acquired from the Department of Pathology, University of Oklahoma Health Sciences Center in accordance with an approved Institutional Review Board protocol.

All of the prostate glands were obtained through radical prostatectomy. A total of 11 cases of CaP were used in this study (Table 1). Prostate tissue that was free of carcinoma was obtained from an additional case (radical cystoprostatectomy) and used as a control. The diagnoses were rendered by four pathologists (BB, CZL, JTY and KMF) and reviewed by one of the pathologists (KMF). Semi-serial paraffin sections were cut at 4–6 µm, dried at 55°C overnight, and stored at –20°C until use. Slides were deparaffinized with xylene and rehydrated in graded ethanol before use as previously described (Lin *et al.* 2004).

Hematoxylin-eosin stain

Deparaffinized and rehydrated slides were stained in hematoxylin and blued in lithium carbonate solution. The slides were then washed, stained with eosin, washed, dehydrated and sealed with Permount Mounting Media (Sigma).

Immunohistochemistry (IHC) of human prostate tissue sections

IHC was performed with a three-step, peroxidase-mediated mechanism as previously described (Fung *et al.* 1995). Briefly, deparaffinized and rehydrated tissue sections were incubated with 0.5% H₂O₂ in methanol for 10 min to block endogenous peroxidase

activity. Antigen retrieval was performed by heating the slides in 10mM citric acid buffer (pH 6.0) in a microwave oven at 1200 W for 20 min. The slides were then washed with 0.1 M Tris–HCl at pH 7.6 (TRIS) for 5 min and then incubated with TRIS containing 10% goat serum to block non-specific binding. Slides were then incubated with AKR1C3 mAb (NP6G6.A6) at a dilution of 1:200 of at 4°C overnight. After washing with TRIS, the slides were incubated with biotinylated goat anti-mouse secondary antibody in TRIS containing 10% goat serum at room temperature for 1 h. Following washes with TRIS, HRP-conjugated streptavidin diluted in TRIS containing 10% goat serum was added to slides and incubated at room temperature for another 40 min. The slides were washed in TRIS for 10 min. A DAB–H₂O₂ substrate was added to the slides and incubated at room temperature for 6 min. Slides were then washed with distilled water and counterstained with hematoxylin. Slides were dehydrated and sealed with Permount Mounting Media for visualization.

Scoring of IHC for AKR1C3-positive cancer cells

The percentage of positively immunoreactive cells with IHC staining was evaluated and graded into five categories: over 75% positive (>75%), 50% positive (50%), 25% positive (25%), 10% positive (10%) and negative (0%).

Results

Detection of AKR1C3 in cultured human prostate cells

We have previously reported the monospecificity of the mAb (NP6G6.A6) and shown that it only recognizes recombinant AKR1C3 among members of the AKR1C family (Lin *et al.* 2004). To demonstrate the ability of this antibody to specifically distinguish AKR1C3 from other cellular proteins, Western blot analysis was performed in total cellular lysates prepared from primary cultures of prostate epithelial cells (normal and CaP) and from androgen-sensitive human prostate cancer LNCaP cells. A single protein species with a molecular mass of approximately 37 kDa was detected in these total cellular protein preparations (Fig. 2B). The recognized protein species had the same size as the recombinant AKR1C3 protein (Lin *et al.* 1997, 2004). To correlate the levels of AKR1C3 protein expression with its transcript expression in the cultured prostate cells, AKR1C3 mRNA was

Figure 2 Comparable levels of AKR1C3 transcript and protein expression in prostate cells. (A) RT-PCR detection of AKR1C3 transcripts. Total RNA was extracted from cultured prostate epithelial cells (PECs) and LNCaP cells and used for reverse transcription. AKR1C3 was amplified using AKR1C3-specific primers. (B) Specificity of anti-AKR1C3 mAb. Total cellular proteins were extracted from cultured prostate cells, electrophoresed on Tris–HCl gels, and transferred to PVDF membranes. Immunodetection of AKR1C3 protein was performed by incubating with mouse anti-human AKR1C3 mAb (clone NP6G6.A6). The immunoblot analysis shows that the mAb can only react with one species of proteins prepared from PECs and LNCaP cells.

amplified using our established RT-PCR procedures using the isoform-specific primer sets for AKR1C3 (Penning *et al.* 2000). The results showed comparable

Figure 3 Expression of type 2 3α -HSD (AKR1C3) in normal prostate tissue. (A) and (B) are taken from the same area at low- and high-magnification respectively. Normal prostatic glands are faintly immunoreactive or negative. The stromal cells and smooth muscle cells are variably immunoreactive. Strong cytoplasmic and nuclear immunoreactivity are demonstrated in both endothelial cells in blood vessels (V) and perineurial cells (arrow). (C) is taken from the prostatic urethra in the verumontanum. There are both cytoplasmic and nuclear immunoreactivity (inset). (D) Consistent immunoreactivity is demonstrated in the seminal vesicles with predominantly cytoplasmic immunoreactivity (inset). Original magnification: (A) $\times 10$; (C) and (D) $\times 20$, (B) $\times 40$; insets $\times 60$.

levels of AKR1C3 protein and transcript expression in these cells (Fig. 2A).

Immunoreactivity of AKR1C3 enzyme protein in normal prostate

The mAb for AKR1C3 was used for immunohistochemical (IHC) detection of AKR1C3 protein in human prostate tissue. Consistently strongly nuclear and cytoplasmic immunoreactivity of the mAb was demonstrated in the endothelial cells and perineurial cells (Fig. 3A and B). Variable immunoreactivity was demonstrated in the stromal cells and smooth muscle cells. The glandular epithelial cells were either faintly immunoreactive or non-immunoreactive. Strong nuclear and cytoplasmic immunoreactivity

was also demonstrated in urothelial (transitional) cells in the prostatic urethra (Fig. 3C). Predominantly cytoplasmic immunoreactivity was demonstrated in the seminal vesicles (Fig. 3D).

Immunoreactivity of AKR1C3 enzyme protein in CaP

Positive immunoreactivity was identified in 9 of 11 cases (81.8%) of CaP (Table 1). In general, a variable percentage of carcinomatous cells in these nine CaP cases was positive. There was no single case in which all the carcinomatous cells were positive but five of the nine cases (55.6%) had positive immunoreactivity in over 50% of the carcinomatous cells (Table 1). Both cytoplasmic and nuclear immunoreactivity were

Figure 4 Expression of type 2 3 α -HSD (AKR1C3) in CaP. (A–C) are taken from Case No. 2. (A) Stained by hematoxylin-eosin stain. Variation in immunoreactivity in the same tumor is demonstrated by the difference between strong immunoreactivity in (B) and negative immunoreactivity in (C). (D) and (E) From Case No. 1. Note the strong immunoreactivity in the cribriform carcinomatous area in (D) and in endothelial cells (arrow). In (E), there is an abrupt transition from strong immunoreactivity in one neoplastic gland to weak immunoreactivity in other neoplastic gland. Note that the nuclear immunoreactivity is still retained in weakly immunoreactive carcinomatous cells (arrow head). (F) From Case No. 7. There are individual immunoreactive carcinomatous cells admixed with negative carcinomatous cells. Original magnification $\times 60$ in all panels.

demonstrated in the carcinomatous cells (Fig. 4A and B). Variation from strong to negative immunoreactivity was demonstrated within the same tumor (Fig. 4B and C) and also among different tumors (Fig. 4D and E). In Cases No. 1 and No. 9, strong immunoreactivities were predominantly found in cribriform areas that corresponded to a Gleason score of 4, while negative immunoreactivity was found in invasive carcinoma with small glands that corresponded to a Gleason score of 3 (Fig. 4D and E). This pattern of expression was not demonstrated in other tumors that had both cribriform and small gland invasive components. In areas with positive immunoreactivity, it was more often the case to demonstrate positive

immunoreactivity in all neoplastic cells within the same gland (Fig. 4B and D). A less common pattern was that individual immunoreactive carcinomatous cells existed within a background of non-immunoreactive carcinomatous cells (Fig. 4F).

Immunoreactivity of AKR1C3 enzyme protein in non-neoplastic conditions

Focally positive immunoreactivity was also demonstrated in benign glands in areas with chronic inflammation (Fig. 5A). Similar focally positive immunoreactivity was also demonstrated in glands with atrophic changes (Fig. 5B). Positive immunoreactivity

Figure 5 Expression of type 2 3α -HSD (AKR1C3) in non-neoplastic pathological conditions. (A) An inflamed area where focal increase in immunoreactivity (arrow) is demonstrated. (B) An atrophic area where focal immunoreactivity (arrowhead) is demonstrated. (C) From a prostate gland close to the verumontanum. Cells undergoing urothelial (transitional) cell metaplasia are strongly immunoreactive but prostatic epithelial cells overlying the urothelial (transitional) cell metaplasia are non-immunoreactive. (D) From an area with basal cell hyperplasia which shows negative immunoreactivity. Note the immunoreactivity in stromal and endothelial cells. Original magnification: (A) $\times 20$; (B–D) $\times 60$.

was demonstrated in glandular epithelium with urothelial (transitional) cell metaplasia (Fig. 5C). In contrast, glandular epithelium with basal cell hyperplasia were non-immunoreactive (Fig. 5D).

Relationship between immunoreactivity of AKR1C3 and AR

IHC staining for AR was performed on sections adjacent to those that had been used for AKR1C3 IHC staining. Both carcinomatous and non-neoplastic prostate gland epithelial cells were immunoreactive for AR. No correlation in staining patterns was observed between AR-positive immunostaining and positive or negative immunoreactivity for AKR1C3 in CaP (Fig. 6).

Discussion

An isoform-specific, mouse anti-human mAb (clone NP6G6.A6) that recognizes AKR1C3 but not other members of the AKR1C family has been developed by us (Lin *et al.* 2004). In this study, we demonstrated that this mAb can recognize AKR1C3 specifically in total cell lysates obtained from primary cultures of human prostate epithelial cells and human prostate cancer LNCaP cells by western blot analysis. One purpose in the report is to demonstrate that the AKR1C3 mAb can recognize a single species of protein in human prostate-derived cells. Detection of AKR1C3 transcripts by RT-PCR in these cells showed parallel expression of AKR1C3 mRNA and protein in human prostate cells.

Figure 6 Correlation of AR and AKR1C3 in prostate tissue. All the panels are taken from the carcinoma in Case No. 5. (A–C) Serial sections taken from the one area while (D–F) are taken from a different area. (A) and (D) Hematoxylin-eosin stained, (B) and (E) are IHC for type 2 3α -HSD (AKR1C3), (C) and (F) are IHC for AR. The histopathological features of both areas are comparable as demonstrated in (A) and (D). Immunoreactivity for type 2 3α -HSD (AKR1C3), however, is positive in one area (B) but negative in another area (E). Nuclear immunoreactivity for AR is demonstrated in both areas (C and F). Original magnification $\times 60$ in all panels.

This antibody was used to study the expression of AKR1C3 in neoplastic and non-neoplastic prostate tissue through IHC. Our results showed cytoplasmic immunoreactivity of AKR1C3 in non-epithelial components of the prostate including endothelial cells, perineurial cells, stromal and smooth muscle cells. Immunoreactivity was also demonstrated in urothelial (transitional) epithelium in the prostatic urethra and also in epithelial cells of seminal vesicles. In contrast, normal prostatic glandular epithelial cells showed only faint or total absence of immunoreactivity with this isoform-specific antibody. Positive cytoplasmic immunoreactivity for AKR1C3 was detected in 9 of 11 CaP

cases. We conclude that AKR1C3 is upregulated in CaP. Increased AKR1C3 immunoreactivity in adenocarcinoma cells is in accord with our previous observation that elevated reductive 3α -HSD enzymes, such as AKR1C2 (Rizner *et al.* 2003) and AKR1C3 (Lin *et al.* 1997) show increased transcript expression in primary cultures of prostate epithelial cells derived from cancerous sections of the prostate.

Surprisingly, we also observed positive nuclear immunoreactivity for AKR1C3. However, this result is consistent with an immunoelectron microscopic study of this enzyme reported by Pelletier *et al.* (2001), although these authors did not emphasize

nuclear localization of this enzyme. It is possible that nuclear AKR1C3 performs a yet unknown function. We have ruled out that the nuclear staining is an artifact of antigen retrieval, since the same staining pattern is observed in the presence and absence of antigen retrieval. It is also possible that the nuclear staining is due to the diffusion of AKR1C3 to the nucleus during the sample handling. However, Pelletier observed the nuclear staining following rapid processing of the prostate tissue.

Co-localization of AKR1C3 and AR was also investigated in normal prostate and CaP specimens. Consistent with previous reports (Chodak *et al.* 1992, El-Alfy *et al.* 1999, Labrie *et al.* 2000b), AR shows a strong nuclei staining pattern. Our data demonstrated that AKR1C3 and AR are co-expressed in the same cell types in prostate tissue, particularly in cases of CaP. However, the increased immunoreactivity of AKR1C3 in CaP cases is not associated with a change in immunoreactivity of AR as evaluated by IHC. Although there appears to be no correlation between AKR1C3 and AR expression in CaP using IHC staining, their co-localization in CaP still supports our hypothesis that AKR1C3 may regulate androgen access to the AR.

Tissue distribution of AKR1C3 has been studied in human reproductive organs including prostate and breast. Using a rabbit polyclonal antibody that was raised against the C-terminal of AKR1C3, El-Alfy *et al.* (1999) demonstrated positive immunoreactivity in stromal and endothelial cells in the prostate. A similar pattern of positive immunoreactivity in prostate stromal and endothelial cells was observed in our study. However, El-Alfy *et al.* demonstrated positive immunoreactivity in the glandular basal cells but not the luminal epithelial cells. In our study, however, most of the luminal and basal epithelial cells of prostatic glands were negative or, at most, faintly immunoreactive. The causes of discrepancies between these two studies are not clear. It may reflect difference in the cross-reactivity of the two antibodies. Our mAb is specific for AKR1C3, while the polyclonal antibody for AKR1C3 may cross-react with AKR1C1, which is expressed in the prostate (D M Bauman and T M Penning, unpublished observations).

Multiple hypotheses exist to explain the elevated AKR1C3 expression in CaP and a role of this enzyme in the pathogenesis of CaP. First, with its type 5 17β -HSD activity, AKR1C3 can generate the local source of potent androgen by converting Δ^4 -dione to testosterone (Labrie *et al.* 2000a). The subsequent intraprostatic accumulation of testosterone can serve as substrate for type 2 5α -reductase, enhance AR

trans-activation activity, and act as a growth advantage for the adenocarcinoma even in the absence of elevated AR protein expression.

Secondly, based on our previous observation that 3α -diol can stimulate prostate cell proliferation through an AR-independent, growth factor-like pathway(s) *in vitro* (Nunlist *et al.* 2004, Zimmerman *et al.* 2004), it is possible that AKR1C3 converts the potent androgen, 5α -DHT, into its cognate metabolite, 3α -diol, and regulates the utilization of AR or growth factor receptor signaling pathways in the prostate. Growing evidence suggests 3α -diol might be an important hormone with its own androgenic effects in hormone target organs through yet undefined pathway(s). It has been suggested that 3α -diol is a more potent androgen than 5α -DHT or testosterone in inducing prostatic hyperplasia in the castrated dog (Walsh & Wilson 1976, Jacobi *et al.* 1978) and may be more effective than 5α -DHT in virilizing the urogenital tract including prostate both in the fetal rat (Schultz & Wilson 1974) and in the fetus of the pouch of female marsupials (Wilson *et al.* 2003a,b, Leihy *et al.* 2004).

Thirdly, via its 11-ketoprostaglandin reductase activity, AKR1C3 catalyzes the 11-keto-reduction of PGD_2 to yield the $PGF_{2\alpha}$ epimer, $9\alpha,11\beta$ - PGF_2 (Desmond *et al.* 2003). This enzymatic function causes AKR1C3 to divert PGD_2 catabolism to $9\alpha,11\beta$ - $PGF_{2\alpha}$ and reduce the accumulation of PGJ_2 , a ligand for PPAR γ receptor (Desmond *et al.* 2003). A functional consequence of PGJ_2 deprivation is that the growth inhibitory effects of $15\Delta PGJ_2$ in CaP are attenuated (Subbarayan *et al.* 2004). This also prevents the downregulation of COX-2, which when elevated can have a pronounced effect on microvessel density and angiogenesis in prostatic tumors (Kirschenbaum *et al.* 2001, Sabichi *et al.* 2004). PGD_2 -induced cell proliferation and PGJ_2 -induced differentiation and programmed cell death have been identified in variety of cell types including prostate cancer cells (Butler *et al.* 2000, Desmond *et al.* 2003, Yee *et al.* 2003, Morosetti *et al.* 2004, Zang *et al.* 2004). Furthermore, the activation of PPAR γ by a synthetic ligand, troglitazone, suppressed AR *trans*-activation activity in prostate cancer cells and serum prostate-specific antigen levels in a patient with CaP (Hisatake *et al.* 2000). Therefore, elevated expression of AKR1C3 may regulate PG metabolism in the prostate and cause the accumulation of the proliferative PG, $9\alpha,11\beta$ - $PGF_{2\alpha}$. AKR1C3 may also be intimately involved in controlling proliferative growth and vascular development in the diseased prostate through this pathway. We are actively studying the relationship between AKR1C3 and PPAR γ expression in CaP.

In our study, elevated AKR1C3 immunoreactivity was also detected in non-neoplastic conditions including chronic inflammation, atrophic changes and urothelial (transitional) cell metaplasia. Lipid metabolism including arachidonic acid and PG has been implicated in these disorders (Heller *et al.* 1998). Furthermore, involvement of PPAR γ has been reported in regulating lipid homeostasis, immune response, cell differentiation and cell proliferation (Heller *et al.* 1998, Bishop-Bailey & Wray 2003, Varley *et al.* 2004). We conclude that upregulation of AKR1C3 as reflected by an increase in immunoreactivity is associated with both neoplastic and non-neoplastic pathological changes in the prostate.

CaP has been recognized as heterogeneous, multifocal cancer that can be induced by multiple carcinogenic factors. In this study, elevated expression of AKR1C3 protein has been demonstrated in the epithelium of the majority of CaP as well as in non-neoplastic processes such as inflammation. As AKR1C3 regulates steroid hormone and PG metabolism in endocrine target tissues, expression of AKR1C3 in CaP implicates a possible role in tumorigenesis of CaP. This possible mechanism awaits elucidation.

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