Effects of the Dual 5 α-Reductase Inhibitor Dutasteride on Apoptosis in Primary Cultures of Prostate Cancer Epithelial Cells and Cell Lines

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Supported by a grant from GlaxoSmithKline and in part from the Irish Cancer Society.

The authors thank Dr. David Healy (University College Dublin School of Medicine and Medical Sciences) for his statistical input and Dr. Catherine Moss (Transcriptomics Unit, University College Dublin Conway Institute of Biomolecular and Biomedical Research) for her expert assistance with the real-time polymerase chain reaction work and analysis.

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Received July 7, 2005; revision received November 24, 2005; accepted January 20, 2006.

BACKGROUND. The profound reduction in serum dihydrotestosterone (DHT) observed with the dual 5 α-reductase inhibitor (5ARI) dutasteride makes it an attractive agent for prostate cancer therapy. The objective of the current study was to determine whether dutasteride would induce apoptosis in a range of prostate epithelial cell lines and primary cultures.

METHODS. Both human prostate androgen-sensitive cell lines (PwR-1E, PNT-2, LNCaP, and PC3[AR2]) and an androgen-independent cell line (PC-3) were grown to confluence. Primary epithelial cells extracted from fresh prostate cancer radical prostatectomy specimens also were grown to confluence under optimal conditions. Total cellular protein was extracted to confirm cytokeratin 18 and antihuman α-methylacyl-CoA racemase (AMACR) expression of the primary cells. Apoptosis was assessed by propidium iodide DNA staining and flow cytometry after 24 hours of culture in from 0 μM to 10 μM of dutasteride.

RESULTS. Dutasteride induced a dose-dependent increase in apoptosis in the androgen-sensitive prostate cell lines PwR-1E, PNT-2, and LNCaP and in the androgen receptor-expressing PC3(AR2) cell line. However, there was no significant apoptosis noted in the parental PC-3 cells. Of 16 primary epithelial cultures that were treated, 7 cultures were induced to undergo apoptosis, and 9 cultures were unresponsive. All primary cultures were positive for cytokeratin 18 expression, confirming their epithelial phenotype. Responder epithelial cells were positive for AMACR expression.

CONCLUSIONS. The results of the current study confirmed that dutasteride differentially induced apoptosis in a subset of prostate cell lines and primary prostate epithelial cells. Understanding the cellular phenotype may indicate susceptible cells. ***Cancer*** **2006;106:2743–52.** © *2006 American Cancer Society.*

KEYWORDS: 5 α-reductase inhibition, dutasteride, apoptosis, prostate cancer, antihuman α-methylacyl-CoA racemase, primary cultures.

Androgens play an important role in controlling the growth of the normal prostate gland and in promoting benign prostatic hyperplasia (BPH) and prostatic carcinoma. The 2 most potent androgens in prostate cancer etiology are testosterone and dihydrotestosterone (DHT).[1] When free testosterone enters prostate epithelial cells, 90% is converted to the main prostatic androgen, DHT.[2,3] This irreversible conversion is catalyzed by 5 α-reductase, a nicotinamide adenine dinucleotide phosphate diaphorase-dependent, 3-oxo-5 α-steroid Δ4-dehydrogenase.[4] There are 2 types of 5 α-reductase enzymes, termed type I (SRD5A1) and type II (SRD5A2), and they are encoded by 2 separate genes.[5]

Because prostate epithelial cells depend on a critical level of

DOI 10.1002/cncr.21938
Published online 15 May 2006 in Wiley InterScience (www.interscience.wiley.com).

androgenic stimulation for growth, function, and survival, androgen-ablation therapy remains the standard treatment for patients with advanced prostate cancer. Although the disease initially may be controlled, progression to the androgen-independent state can occur within 2 to 5 years.[6] These androgen-independent cell populations resist the apoptotic effects of androgen-withdrawal therapy and survive.[7]

It has been demonstrated that androgen-deprivation therapy decreases the prevalence and extent of prostatic intraepithelial neoplasia lesions, which are the most likely precursor of prostatic carcinoma in the peripheral zone,[8] suggesting that this form of treatment may play a role in chemoprevention.[9] Although, in the Prostate Cancer Prevention Trial,[10] a possible chemopreventative role was found for the SRD5A2 inhibitor finasteride, because there was a 24.8% reduction in the period prevalence of prostate cancer reported, there was a concomitant increase in the proportion of tumors with high-grade disease in the treatment arm (6.4%) compared with tumors in the placebo arm (5.1%). The profound reduction in serum DHT observed with the dual 5 α-reductase inhibitor (5ARI) dutasteride has led to the initiation of the Reduction by Dutasteride of Prostate Cancer Events study, which is examining the effect of dutasteride on the prevention of prostate cancer. The possible chemopreventative role of dutasteride will not be known until that trial is complete in 2008.

It has been demonstrated that dutasteride blocks the activation of the androgen receptor (AR) with the consequences of decreased proliferation and increased death of LNCaP cells.[11] This recently was confirmed by Schmidt et al.,[12] who also showed that dutasteride effectively inhibited both the viability and proliferation of LNCaP cells within 48 hours after treatment with concentrations of dutasteride from 1 μM to 10 μM. Their study revealed both the differential expression of several groups of genes and the dramatically altered expression of genes involved in the metabolic, cell cycle, and apoptotic pathways and, as expected, in the androgen-signaling pathways. The expression levels of several messenger RNA (mRNA) gene products involved in the Fas ligand/tumor necrosis factor α apoptotic pathway, such as TNF-receptor-associated death domain (TRADD), caspase 7, and caspase 8, were increased. Genes that are involved in resistance to apoptosis, such as *BIRC1* (baculoviral inhibitor of apoptosis proteins repeat-containing 1), also demonstrated high levels of expression. Overall, investigations in LNCaP cells (which contain the T877A mutation in the AR[13]) and PC-3 cells[11,12] have suggested the possibility of a second pathway for high-dose dutasteride-induced cell death, independent of the AR. For these reasons, we wanted to determine the effect of short-term treatment with dutasteride on a range of different prostate cell lines and primary prostate cancer epithelial cells.

MATERIALS AND METHODS

Materials

RPMI-1640, WAJC 404 medium, penicillin/streptomycin, L-glutamine, heat-inactivated fetal bovine serum (FBS), and keratinocyte medium with supplements were purchased from GIBCO Life Technologies Ltd. (Cambridge, U.K.). Zinc-stabilized insulin, dexamethazone, hydrocortisone, and cholera toxin were obtained from Sigma (Dublin, Ireland). Collagenase Type I was acquired from Lagan Bach services (Bray, Ireland).

The primary antibodies used were mouse antihuman cytokeratin 8 (CK8)/CK18 (Santa Cruz Biotechnology Inc., Santa Cruz, CA) and rabbit antihuman α-methylacyl-CoA racemase (AMACR) (Biologo, Kronshagen, Germany). Blots routinely were stained for equal protein loading by using mouse anti-β-actin monoclonal antibody (Sigma Chemical Company, Saint Louis, MO).

Cell lines and primary cultures (65-70% confluence) were treated with dutasteride (0.1-10 μM) (GlaxoSmithKline, Research Triangle Park, NC). Dutasteride (25 mM) was dissolved in dimethyl sulfoxide ($\leq$0.1% final concentration) prior to cell treatment.

Total RNA was extracted from primary cultures with Trizol according to the manufacturer's instructions (Molecular Research Center, Inc., OH). All materials related to combinational DNA (cDNA) synthesis were purchased from Invitrogen (Dublin, Ireland).

Real-time PCR TaqMan assay was used to quantitate the relative gene expression levels of SRD5A1, SRD5A2, and AR. These assays were purchased as gene-expression assays from the Applied Biosystems website (available at URL: www.appliedbiosystems.com [accessed January 10, 2006], assay no. Hs00602694_mH for SRD5A1, Hs00165843_m1 for SRD5A2, and Hs00171172_m1 for AR). The ribosomal 18S gene was used as an endogenous control for normalization of the target genes. 18S primers and probe were supplied as a predeveloped assay reagent (P/N 4310893E; Applied Biosystems, Warrington, UK). Taqman Universal polymerase chain reaction (PCR) Master Mix (P/N 4304437) was supplied by Applied Biosystems, and we employed the concentrations and volumes recommended by the supplier. cDNA was amplified on the ABI 7900HT Sequence Detection System.

Methods

Cell culture

The human prostate androgen-sensitive PwR-1E, PNT-2, LNCaP cell lines and the androgen-independent prostate cell line PC-3 were purchased from the American TypeCulture Collection (Rockville, MD). The PC3(AR2) (AR-expressing) cell line[14] was a kind gift from Theodore Brown (University of Toronto, Toronto, Ontario, Canada). The benign prostatic cell lines PwR-1E and PNT2 were cultured in keratinocyte medium (with 50 μg/mL of bovine pituitary extract, 5 ng/mL of epidermal growth factor, 50 U/mL of penicillin, and 50 μg/mL of streptomycin) and in RPMI-1640 medium (with 10% heat-inactivated FBS, 50 U/mL of penicillin, 50 μg/mL of streptomycin, and 2 mM of L-glutamine), respectively. The LNCaP cell line was cultured in RPMI-1640 with 10% heat-inactivated FBS, 50 U/mL of penicillin, 50 μg/mL of streptomycin, 0.5% glucose, and 2 mM of L-glutamine. The PC-3 cell line was cultured in RPMI-1640 medium with 10% heat-inactivated FBS, 50 U/mL of penicillin, 50 μg/mL of streptomycin, and 10 mM of L-glutamine. The PC3(AR2) cell line was cultured in RPMI-1640 medium with 5% heat-inactivated FBS, 2 mM of L-glutamine, and 100 μg/mL of Hygromycin B (Calbiochem, Nottingham, U.K.).

Primary epithelial cell lines also were established by using a previously described protocol.[15] Tissue was obtained after informed consent from patients who underwent radical prostatectomy in the Mater Misericordiae University Hospital, Dublin. Under pathologic direction, both tumor areas and benign areas were sectioned and confirmed by the adjacent surrounding histology. These epithelial cells were cultured in WAJC 404 medium (GIBCO) supplemented with 10% heat-inactivated FBS, 25 U/mL of penicillin, 25 μg/mL of streptomycin, 28 mM of N-2-hydroxyethyl piperazine-N′-2-ethane sulphonate, 14.2 mM of $NaHCO_3$, 20 ng/mL of cholera toxin, 1 μM of dexamethazone, 25 ng/mL of zinc-stabilized insulin, 2.5 μM of hydrocortisone, and 250 μg/mL of amphotericin B. Fibroblast aggregates, which were removed from the supernatant fluid by an additional centrifugation step at 2000 revolutions per minute for 10 minutes, then were grown in RPMI-1640 medium supplemented with 10% heat-inactivated FBS, 50 U/mL of penicillin, 50 μg/mL of streptomycin, 10 mM of L-glutamine, and 500 μg/mL of amphotericin B. The established primary cell lines were sustained only from 6 to 8 weeks and were passaged either 25-cm^3 or 75-cm^3, vented tissue-culture flasks or in 24-well plates at 37°C in a humidified atmosphere of 5% carbon dioxide.

Quantification of cell apoptosis, viability, and cell cycle

Apoptosis was quantified as the percentage of cells with hypodiploid DNA assessed by propidium iodide (PI) incorporation, as described previously.[15] Approximately 1×10^6 cells were centrifuged at 1100 revolutions per minute for 5 minutes; then, they were resuspended gently in 400 μL of a hypotonic fluorochrome solution consisting of 50 μg/mL of PI, 3.4 mM of sodium citrate, 1 mM of Tris, 0.1 mM of ethylenediamine tetraacetic acid (EDTA), and 0.1% Triton X-100. The samples were stored in the dark at 4°C for between 15 minutes and 20 minutes prior to analysis using an Epics XL-MCL Coulter ELITE cytofluorometer (BD BioSciences, Franklin Lakes, NJ). At least 5000 events were collected and analyzed. Decreased PI intensity represents increased double-stranded DNA cleavage. Early necrotic cells do not have double-stranded breaks and have high PI staining, as do normal cells. PI viability assays also were performed to distinguish between the intact cellular membranes of apoptotic cells and the disrupted membranes of necrotic cells. Apoptotic cell nuclei were distinguished from normal nuclei by their hypodiploid DNA. Cellular debris was excluded from the analysis by raising the forward threshold. All measurements were performed under the same instrument settings. For cell cycle analysis, in total, 5000 events were gated on PI intensity into G_1/S-phase and G_2M-phase. The proportion of cells that entered G_2M-phase are expressed as a percentage of total cells gated across G_1/S-phase and G_2M-phase.

Western blot analysis

Total protein was isolated after treatment using the NP-40 protein-isolation solution[16] (0.5% NP-40; 10 mM of Tris [pH 8.0], 60 mM of KCl, 1mM of EDTA [pH 8.0], 1 mM of dithiothrietol [DTT], 10 mM of phenyl methyl sulfonyl fluoride, 1 μM each of leupeptin and aprotinin, and 2 μM of pepstatin). Protein content was assayed using the DC protein assay kit (BioRad, Inc., Cambridge, MA). Protein (50 μg) was run on a 12% sodium dodecyl sulfate-polyacrylamide gradient gel, which was transferred electrophoretically to Immobilon-P (Millipore, Bedford, MA). Equal protein loading was confirmed routinely by staining for β-actin and Coomassie blue. Blots were blocked with 1% bovine serum albumin/Tris-buffered saline and 0.1% Tween-20 for 1 hour at room temperature, after which they were incubated in either the AMACR (1:500 dilution) or CK8/CK18 primary antibody (1:500 dilution). After washing, membranes were incubated with either horseradish peroxidase-conjugated antimouse immunoglobulin G (Cell Signaling Laboratories, Beverly,

MA) or goat antirabbit immunoglobulin G (BD Transduction Laboratories, Oxford, U.K.) at a 1:5000 dilution for 1 hour. Blots were developed by using an enhanced-chemiluminescence substrate system for the detection of horseradish peroxidase (Amersham Biosciences, UK Ltd., Newcastle-upon-Tyne, U.K.).

cDNA synthesis from template RNA

After total RNA extraction, 1 μg of total RNA was DNase-treated by incubating with 1 $\times$ DNase I reaction buffer and 0.1 U DNase I (amplification grade). The reaction was terminated by the addition of 2 mM of EDTA followed by heat inactivation for 10 minutes at 65°C. The prepared template was then incubated with 1 $\times$ first-strand buffer, 10 mM of DTT, 0.5 mM of dinucleotide triphosphate mix, and 0.015 μg of random primers with 20 U of SuperScript II RNase H-Reverse Transcriptase. One cycle of PCR was performed as follows: a primer-annealing step for 10 minutes at 25°C, reverse transcription for 50 minutes at 42°C, and an enzyme-inactivation step for 15 minutes at 70°C.

Real-time reverse transcriptase-PCR

PCR reactions in duplicate for SRD5A1 and AR were set up in separate tubes with Taqman Universal PCR Master Mix, after which cDNA was amplified at default thermal cycling conditions: 2 minutes at 50°C, 10 minutes at 95°C for enzyme activation followed by 40 cycles of 15 seconds at 95°C for denaturation, and 1 minute at 60°C for annealing and extension. Results were analyzed by using the Δ Cycle Threshold method of analysis.

Statistical Analysis

Statistical analysis was performed using a 1-way analysis of variance (ANOVA) with Student–Newman correction. Analysis was also performed, as indicated, by using a 1-tailed or 2-tailed Student *t* test for unpaired data. Significance was assumed for values of $P \leq .05$. Results are expressed as mean $\pm$ standard deviation.

RESULTS

Effects of Dutasteride on Prostate Cancer Epithelial Cell Apoptosis

First, we undertook time course studies on the effects of dutasteride on apoptosis in a variety of prostate cell lines, including the androgen-sensitive PwR-1E, PNT2, LNCaP, PC3(AR2) cell lines and the androgen-independent PC-3 cell line. Based on the ability of dutasteride to induce apoptosis, the cell lines could be divided into responders or nonresponders (Table 1). It is noteworthy that the PC3 cell line was unresponsive to dutasteride treatment, whereas the AR-positive PC3(AR2) cell line responded. The LNCaP and PC3(AR2) cell lines remained responsive with treatment out to 4 days and 5 days, respectively; however, the PC3 cell line remained unresponsive with treatment up to 4 days (data not shown).

TABLE 1
Time Course Study of the Effects of Dutasteride on Prostate Cell Apoptosis*

Cell Line	Dutasteride, (μM)	% Apoptosis		
		12 Hours	24 Hours	48 Hours
Responders				
PwR-1E	0	9.1 ± 3.0	9.7 ± 6.2	9.3 ± 3.0
PwR-1E	10	14.0 ± 8.7	18.0 ± 5.8†	23.1 ± 9.0†
PNT2	0	7.3 ± 3.56	5.8 ± 0.7	11.0 ± 3.9
PNT2	10	18.7 ± 0.6†	25.2 ± 4.9†	29.6 ± 9.2†
LNCaP	0	7.8 ± 0.5	11.8 ± 0.8	5.5 ± 6.8
LNCaP	10	9.2 ± 1.6	14.8 ± 0.4†	18.8 ± 1.3†
PC3 (AR2)	0	4.1 ± 0.6	4.1 ± 1.5	6.9 ± 1.7
PC3 (AR2)	10	8.6 ± 6.5	8.4 ± 4.7	20.5 ± 4.18†
Nonresponder				
PC3	0	7.6 ± 1.1	3.5 ± 1.7	8.9 ± 1.0
PC3	10	6.4 ± 1.0	5.4 ± 2.2	10.2 ± 2.6

* The prostate androgen-sensitive PwR-1E, PNT2, LNCaP, the androgen receptor-expressing PC3 (AR2) and the androgen-independent PC3 cell line were treated with either with 0 μm of dutasteride (vehicle control at a final dimethyl sulfoxide concentration of <0.1%) or with 10 μM dutasteride over 48 hours and were assessed for apoptosis by propidium iodide DNA staining and flow cytometry. The results are presented as the mean percentage apoptosis ± standard deviation (average, 3 experiments). Statistical analyses were performed using Student *t* tests for paired data.
† $P \leq .05$ versus control.

Next, dose-response curves were constructed with a dutasteride-responsive cell line (PwR-1E) and an unresponsive cell line (PC3), which were treated with increasing concentrations of dutasteride (0-10 μM). Dutasteride at 10 μM resulted in a significant ($P \leq .05$) induction of apoptosis after 24 hours in the PwR-1E cell line (Fig. 1) with no alteration in cell necrosis (data not shown). However, PC-3 cells did not undergo any significant dose-dependent increase in apoptosis (Fig. 1) or necrosis (data not shown) when they were treated for 24 hours.

Then, we assessed the effects of dutasteride in established, first-passage, primary prostate cancer cultures. Treatment of patient primary prostate cancer cells had differential responses to the effects of dutasteride. An ANOVA of all treated patient samples ($n = 16$ samples) showed that there was no significant increase in dutasteride-induced apoptosis after incubation for 24 hours (Fig. 2A). However, further analysis revealed 2 specific groups: samples that were classified as either dutasteride-responders ($n = 7$ samples) (Fig. 2B) or nonresponders ($n = 9$ samples) (Fig. 2C). Responders were differentiated from nonresponders based on a 50% induction of apoptosis at 5 μM and 10

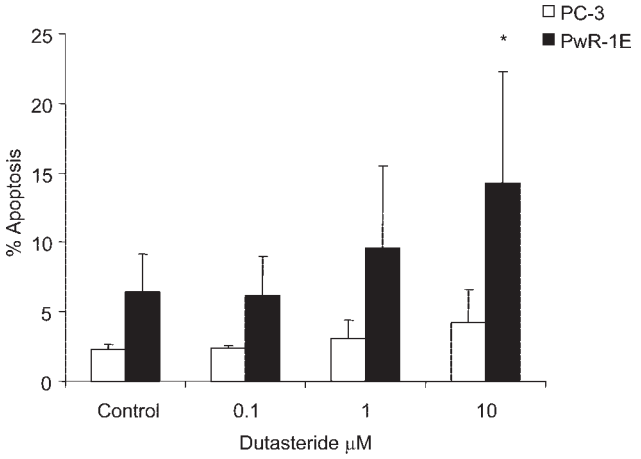


FIGURE 1. The effects of dutasteride are illustrated on PC-3 (androgen-independent; androgen receptor [AR]-negative) and PwR-1E (androgen-dependent; AR-positive) cell apoptosis. PC-3 and PwR-1E cells were treated with 0 μm, 0.1 μm, 1 μm, and 10 μm dutasteride for 24 hours. The cells then were assessed for the percent apoptosis by using propidium iodide DNA staining and were analyzed by flow cytometry. Results shown are the mean $\pm$ standard deviation of 2 experiments. Statistical analyses were performed using a 1-way analysis of variance. An asterisk indicates $P \leq .05$ versus control.

μM dutasteride. Because we had predicted that dutasteride would induce apoptosis, a direct comparison was made between the rates of apoptosis after 5 μM and 10 μM dutasteride treatment between our 2 groups. Responders showed induction of apoptosis in response to 5 μM dutasteride compared with nonresponders, but the difference was not significant. However, responders showed a significant difference ($P = .037$; unpaired; 1-tailed Student t test) in the apoptotic rate to 10 μM dutasteride compared with nonresponders.

Effects of Dutasteride on Prostate Cell Cycle Progression

To ascertain whether dutasteride affected the passage of treated prostate cells through the cell cycle, we assessed cell cycle profiles of the dutasteride-responsive LNCaP cell line at 48 hours posttreatment and calculated the percentage of cells that progressed to G_2M-phase. There was no significant decrease in G_2M-phase induced by dutasteride in the LNCaP cells. Cell cycle analysis in primary prostate epithelial cells demonstrated that dutasteride had no significant effect on the progression to G_2M-phase in either the responder group or the nonresponder group (Fig. 3B).

Characterization of Primary Prostate Cancer Cells

Characterization of the primary tumor from which cells were isolated

To ascertain the differences between the responder and nonresponder isolated prostate epithelial cells, first, we first assessed characteristics of the primary tumor from which the cells were derived. The preoperative prostate-specific antigen (PSA) levels did not differ significantly in patients samples between either the responders (6.2 ± 1.6 ng/mL) or the nonresponders (8.5 ± 5.2 ng/mL). However, there was a significant increase ($P = .04$; unpaired, 2-tailed Student t test) in the Gleason grade of primary tumors from which the responder cells (6.4 ± 1.20) were cultured compared with nonresponders (5.2 ± 0.83). Despite this finding, the clinical significance would have to be preserved in a larger study.

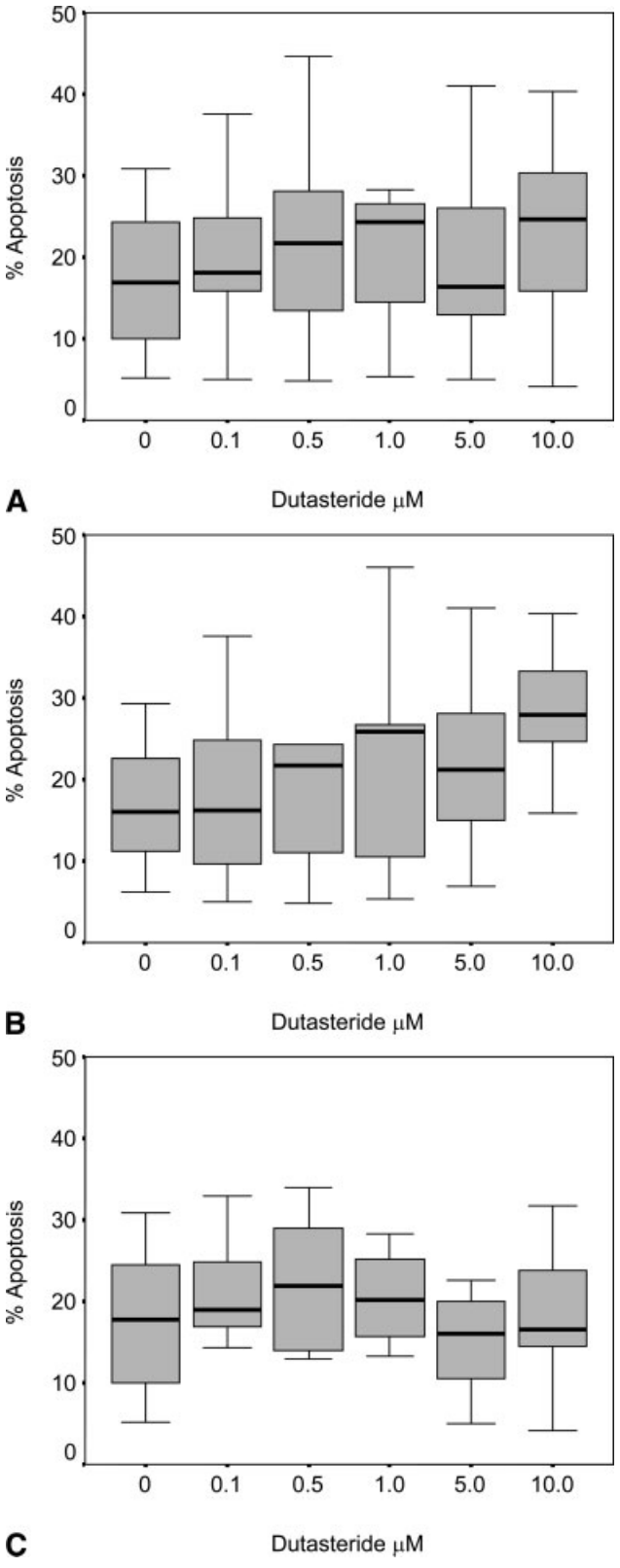
Characterization of cells grown from the tumor

Next, we determined any difference in the phenotypic characteristics of the derived primary epithelial cells. Morphologic assessment demonstrated typical epithelial cell characteristics of a single population of cells with no stromal contamination (Fig. 4A). To confirm their differentiated, luminal phenotype, primary cultures were assessed for the expression of the epithelial cell marker CK18. All tumor cells that were grown from the primary tissue were positive for CK18, which was indicative of a secretory phenotype (Fig. 4B). Because advanced prostate cancer cells have increased resistance to apoptosis, the expression of AMACR was assessed to determine the presence of a malignant phenotype. Figure 4C shows that there was expression of the AMACR protein among the dutasteride-responsive patient samples compared with nonresponsive patient samples.

Analysis of Primary Prostate Cell 5 α-Reductase and AR Expression

To ascertain whether the observed differences in apoptotic response to dutasteride between our responder and nonresponder groups could be explained by differences in either of the 5 α-reductase isoenzymes, we assessed the expression of both Type I (SRD5A1) and Type II (SRD5A2) at the transcriptional level by using specific, real-time PCR Taqman assays. Figure 5A shows the SRD5A1 expression levels for 3 responder and nonresponder patient samples without a significant difference (2-tailed Student t test for unpaired data). SRD5A2 was not detected in any of the same duplicate samples.

The levels of AR expression also were assessed by using a specific, real-time PCR Taqman assay in the same responder and nonresponder samples that were assayed for SRD5A1. Again, there was no significant difference (2-tailed Student t test for unpaired data) in expression between the 2 groups (Fig. 5B).



DISCUSSION

Prostate cancer incidence continues to rise, and new strategies for its prevention and treatment are required. Dutasteride is a Type I and II 5ARI. Recent studies have shown that it induces apoptosis directly in both the LNCaP and PC-3 prostate cancer cells lines independent of the AR.[11,12]

The current study was designed to determine the apoptotic effects of dutasteride further in a range of both androgen-sensitive and androgen-independent prostate cancer cell lines, including primary prostate cancer cell cultures that were isolated from patients who underwent radical prostatectomy. Our novel findings show that dutasteride exhibited differential effects in a range of prostate cell lines that were divided into responders and nonresponders. For instance, 10 μM dutasteride treatment of the PwR-1E cell line (a nonmalignant prostate cell phenotype) demonstrated a significant induction of apoptosis as early as 24 hours after treatment. We also verified the published findings by Lazier et al.[11] and Schmidt et al.,[12] who demonstrated a dose-dependent decrease in cell numbers of the androgen-sensitive LNCaP cell line with similar concentrations of dutasteride up to 96 hours. We have shown that the PC3(AR2) AR-positive cell line responded to dutasteride treatment; however, when the androgen-independent PC-3 cell line was treated for the same duration, there was no significant apoptotic induction. These findings differ from those of Lazier et al.,[11] who demonstrated an effect at 9 days of incubation. However, long-term treatment proved difficult because of cell over-growth and death attributed to confluency. From our study, we conclude that the dutasteride-induced effects are AR-dependent. However, Lazier et al. demonstrated that, although binding of the LNCaP cell AR by the

FIGURE 2. The effects of dutasteride are illustrated on primary prostate cancer epithelial cell apoptosis for (A) all patients ($n = 16$), (B) for responders ($n = 7$), and (C) for nonresponders ($n = 9$). Primary prostate cancer epithelial cells were derived from individual prostate samples that were collected at the time of radical prostatectomy. Primary epithelial cells were incubated with different concentrations of dutasteride (range, 0-10 μM) and were assessed for apoptosis after 24 hours by using propidium iodide DNA staining followed by flow cytometry. Vertical bars of box plots indicate the extreme upper and lower values, and horizontal boundaries of boxes represent the 1st and 3rd quartiles, respectively. Statistical analysis was performed using a 1-way analysis of variance. There was no significant difference ($P \leq .05$) noted between control and any concentration of dutasteride within each group. However, further analysis demonstrated a significant difference between responders and nonresponders ($P = .037$) after treatment with 10 μM dutasteride (1-tailed Student t test for unpaired data).

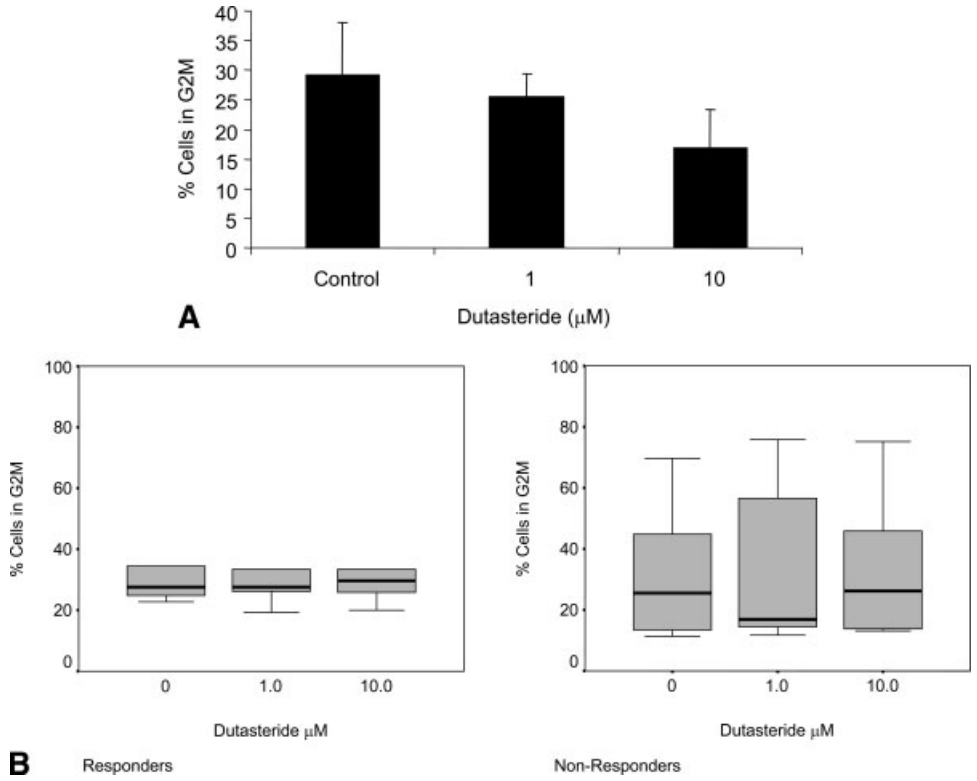


FIGURE 3. The effects of dutasteride on cell cycle progression are illustrated. (A) The effects of dutasteride are shown on the percentage of cells that entered G_2M-phase. Dutasteride-responsive LNCaP cells were treated with control, 1 μM, and 10 μM of dutasteride for 48 hours; then, the percentage of cells that entered G2M phase were assessed using flow cytometry. The results are expressed as the mean $\pm$ the standard deviation of 4 experiments. The decrease in the percentage of cells that entered G_2M-phase was not significant. (B) The effects of dutasteride are shown on the percentage of primary prostate cancer epithelial cells that entered cycle G_2M-phase. Treated primary prostate epithelial cells were from responders ($n = 7$ patients) and nonresponders ($n = 9$ patients). Statistical analysis was performed using a 1-way analysis of variance. There was no significant difference found between control, 1 μM, or 10 μM concentrations of dutasteride.

synthetic androgen R1881 partially protected the cells from dutasteride-induced apoptosis; it did not abolish apoptosis completely, suggesting that an alternate pathway, independent of a functioning AR, must mediate the apoptotic effects of dutasteride.

In the analysis of all 16 primary cultures generated, we demonstrated that dutasteride did not have any significant apoptotic induction; however, further analysis of the results demonstrated that patients could be stratified into 2 cohorts: responders (with 50% induction of apoptosis) and nonresponders. Because each primary represents a separate cell line, characterizing the phenotype of these cells may help in the identification of a dutasteride-susceptible phenotype. First, morphology of the isolated cultured cells confirmed a fibroblast-free, epithelial cell phenotype. Further characterization confirmed a secretory phenotype, as evidenced by consistent positivity for CK18.[17] The next characterization step was to determine the extent of tumor development. There was no correlation between the original PSA serum level and the cells' response to dutasteride. However, there was a significant increase in the original tumor Gleason scores of the responders compared with the nonresponders. The clinical significance of this finding will need to be pursued as part of a larger study.

Previous work on AMACR, which is a metabolic enzyme,[18] demonstrated consistently elevated expression of AMACR mRNA in prostatic carcinoma[19] compared with other cancers. However, it also has been demonstrated that AMACR expression is down-regulated at both the mRNA level and the protein level in hormone-refractory metastatic prostate cancer.[20,21] In the current study, there was an increase in AMACR protein expression that was correlated with Gleason scores in the responder samples only (Fig. 4C). This finding is in contrast to our predicted result, which indicated that nonresponders would have a more aggressive, androgen-independent disease than responders, but this was not the case. However, unfortunately, the finding raises the possibility that the primary cells grown from low-grade tumors were

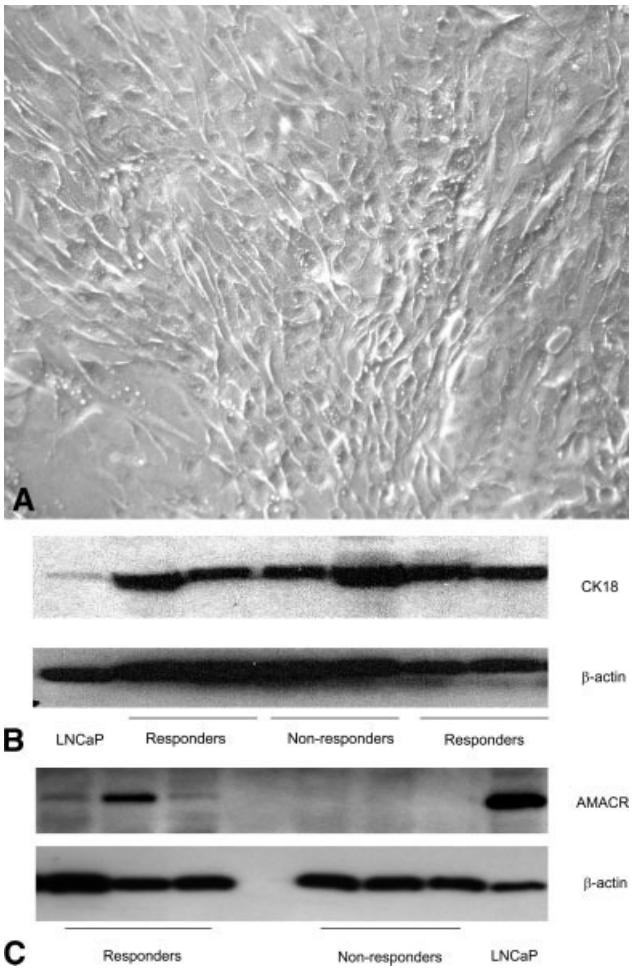


FIGURE 4. Prostate epithelial cells are characterized. (A) A light-microscope image (original magnification, ×10) of isolated primary prostate epithelial cell culture from a representative patient sample showing the characteristic "cobble-stone" morphology associated with epithelial cell cultures. There was no morphologic evidence of contaminating fibroblast/stromal cells. (B) Primary prostate cancer epithelial cell cytokeratin 18 (CK18) expression is characterized. Total cellular protein (50 μg), was extracted from primary prostate cancer epithelial cells, analyzed by Western blot analysis, and probed with mouse anti-CK18 antibody. Total cellular protein extracted from untreated LNCaP cells acted as a positive control. Equal loading was confirmed by mouse anti-β-actin monoclonal antibody. Western blots represent the results from 3 responders and 3 nonresponders. (C) Primary prostate cancer epithelial cell α-methylacyl-CoA racemase (AMACR/P504S) expression is characterized. Total cellular protein (50 μg) was extracted from primary prostate cancer epithelial cells, analyzed by Western blot analysis, and probed with rabbit anti-P504S antibody. Protein extracted from untreated LNCaP cells acted as a positive control. Equal loading was confirmed by mouse anti-β-actin monoclonal antibody. Western blots represent the results from 3 responders and 3 nonresponders.

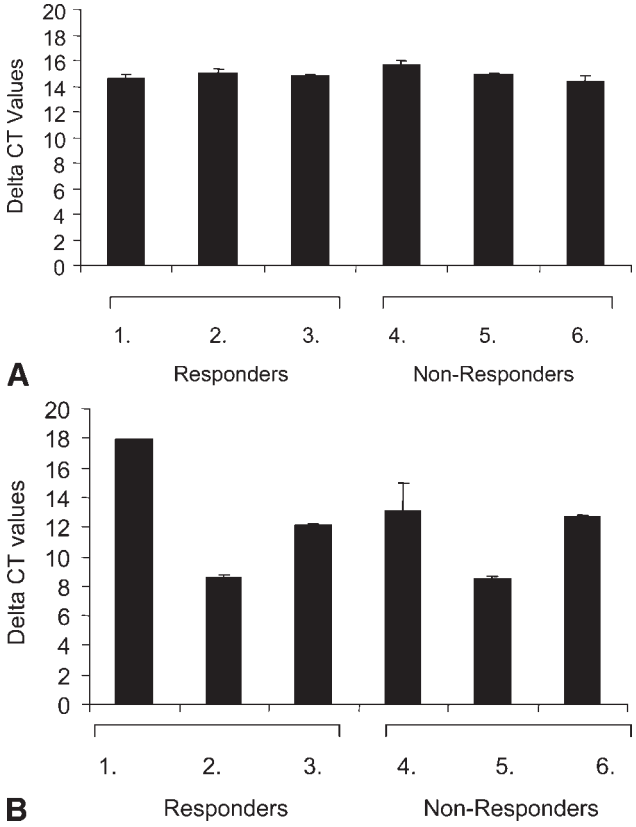


FIGURE 5. Real-time polymerase chain reaction (PCR) analysis is illustrated in prostate epithelial cell cultures. Quantification of primary prostate cancer epithelial cell (A) 5 α-reductase type I (SRD5A1) and (B) androgen receptor (AR) was determined by specific, real-time PCR TaqMan assays. Analysis was performed on combinational DNA samples from 3 representative responders and 3 representative nonresponders. Data were normalized relative to the ribosomal 18S endogenous control and are represented as the average Δ cycle threshold (ΔCT) of each sample in duplicate $\pm$ standard deviation.

more basal in phenotype despite their positive CK18 status and, thus, would not require DHT for survival. Further analysis of other prostate luminal and basal phenotype markers of interest would be the production of PSA and prostatic acid phosphatase (PAP) for luminal cells and the production of basal-specific cytokeratins (CK5, CK14, and CD44).[17]

We also assessed the expression of Type I (SRD5A1) and Type II (SRD5A2) 5 α-reductase at the transcriptional level in dutasteride responders and nonresponders. SRD5A1 was expressed in responder and nonresponder samples; however, SRD5A2 was not expressed consistently. Previously published reports[5,22] have demonstrated decreased expression of 5 α-reductase type II in prostate cancer specimens by microarray, reverse transcriptase-PCR analysis,[5] and immunostaining.[22] Thomas et al.[23] demonstrated high immunoreactivity for 5 α-reductase Type I in 28% of

prostate tumor epithelia specimens. Differences in the expression of 5 α-reductase isoenzymes in our samples also may reflect the primary nature of the model used. Bayne et al.[24] reported that, for BPH-derived epithelial cultures, SRD5A2 transcripts were detectable by reverse transcriptase-PCR at Day 10 of culture, but this expression was absent from cultures that were incubated for 14 days. However, when epithelial cultures were incubated with prostate fibroblast-conditioned medium, Type II mRNA expression was restored, which is suggestive of a fibroblast-specific factor that induces the transcription of SRD5A2 in primary prostate epithelia. Therefore, the expression of SRD5A2 in prostate epithelia appears to depend on the duration of culturing and/or fibroblast presence.

AR expression levels also were assessed in our cultures by real-time PCR. The same responder and nonresponder samples that expressed SRD5A1 also expressed AR. Because fibroblasts are required for maintaining the expression of AR in long-term cultures,[25,26] we cultured the cells only for 6 to 8 weeks, which previously has been shown to maintain AR mRNA expression.[27]

The similar expression of both SRD5A1 and the AR between our responder and nonresponder cultures cannot account for the difference in apoptotic response to dutasteride treatment. Other underlying differences possibly may determine an individual's sensitivity to dutasteride treatment, such as steroid hormone metabolism. Metabolic enzymes, other than or in addition to 5 α-reductase, influence the local availability of testosterone and DHT to cancer cells. Such enzymes may include aldo-keto reductases (AKRs) like AKR1C, 17β-hydroxysteroid dehydrogenase (HSD) Type 5, and/or 17β-HSD Type 10.[28] In addition, with increasing age, the ratio of estrogen to testosterone increases, thus leading to a gradual increase in the estrogenic dominance of the local environment.[29] Changes in the expression of estrogen receptors (ERs) ERα and ERβ also have been reported during the development of prostate cancer and at different stages of prostate cancer.[30]

Why the higher Gleason score/AMACR-positive cells still were responsive to dutasteride is unknown, but this may represent androgen-responsive disease among responders who have not yet progressed to the hormone-refractory phenotype. Furthermore, it is important to note that none of these patients had been treated previously with androgen-ablative therapies. Previous analysis of AMACR expression by immunohistochemical[19] and cDNA microarray studies[20,21] of benign prostate, BPH, localized hormone-naive prostate cancer, and hormone-refractory metastatic prostate cancer demonstrated a significantly stronger expression among the clinically localized samples compared with benign tissues. However, a decrease was observed for metastatic disease.[20,21] It also is noteworthy that patient AMACR expression was independent of hormone therapy, because both hormone-refractory and untreated metastases expressed AMACR, indicating that the regulation of its expression is androgen independent.[21] Western blot analysis of AMACR expression among the PC3, DU145, RWPE, and LNCaP cell lines revealed that both of the hormone-independent cell lines exhibited lower constitutive expression compared with the hormone-responsive LNCaP cells, whereas the benign cell line RWPE showed near absent expression.[20]

To start exploring the mechanism that contributes to apoptotic responsiveness, we assessed whether dutasteride induced any changes in cell cycle progression. There were no significant changes in the percentages of treated primary responder and nonresponder epithelial cells that progressed to G_2M-phase of the cell cycle after treatment for 24 hours. In a recent study, Festuccia et al.[31] showed that dual inhibition of both 5 α-reductase enzymes by MK906 (Finasteride) and MK386 in primary cultures of prostate cancer cells (that contained both fibroblast/stromal cells and epithelial cells) induced several morphologic changes and demonstrated growth inhibition. In addition, inhibition of growth also was observed in the same study in LNCaP cells that were cocultured with prostate cancer fibroblasts and in LNCaP cells that were cocultured with fibroblasts or stroma derived from BPH specimens. In the current study, we showed that the proportion of cells entering the G_2M-phase of the cell cycle was reduced in LNCaP cells that were treated with both 1 μM and 10 μM dutasteride at 48 hours.

The results of the current study demonstrated that dutasteride effectively induced apoptosis in a subset of prostate cell lines. The cells that were responsive to the treatment included the androgen-sensitive PwR-1E, PNT2, LNCaP, and PC3(AR2) cell lines, but the androgen-independent PC3 cells were unresponsive. We also demonstrated that dutasteride induced significant apoptosis at the 10-μM concentration in the androgen-dependent PwR-1E cell line, but not in the androgen-independent PC3 cell line, after treatment for 24 hours. Furthermore, the results of the current study revealed the existence of 2 groups of prostate epithelial cells that were grown from tissue collected from patients who underwent radical prostatectomy for localized disease: some patients responded to the effects of dutasteride, whereas others did not. This is an important observation, because the selective identification of patients who are most likely to respond to

dutasteride treatment will be of paramount importance in determining the role of dutasteride either as chemoprevention or as treatment for prostate cancer. Clinical criteria, such as Gleason scoring, and the use of cellular markers of malignancy, such as AMACR (among others), will aid in the identification of those patients who are most likely to benefit from dutasteride therapy. The global development of a standard panel of markers for use in such patient selection will be vital to realize maximally successful treatment with dutasteride or any other agent for prostate cancer.

REFERENCES

1. Long BJ, Grigoryev DN, Nnane IP, Liu Y, Ling YZ, Brodie AM. Antiandrogenic effects of novel androgen synthesis inhibitors on hormone-dependent prostate cancer. *Cancer Res.* 2000;60:6630-6640.
2. Feldman BJ, Feldman D. The development of androgen-independent prostate cancer *Nat Rev Cancer.* 2001;1:34-45.
3. Foley CL, Kirby RS. 5 Alpha-reductase inhibitors: what's new? *Curr Opin Urol.* 2003;13:31-37.
4. Makridakis NM, di Salle E, Reichardt JKV. Biochemical and pharmacogenetic dissection of human steroid 5α-reductase type II. *Pharmacogenetics.* 2000;10:407-413.
5. Luo JT, Dunn TA, Ewing CM, Walsh PC, Isaacs WB. Decreased gene expression of steroid 5 alpha-reductase 2 in human prostate cancer: implications for finasteride therapy of prostate carcinoma. *Prostate.* 2003;57:134-139.
6. Iversen P. Current topics in the treatment of hormone-refractory prostate cancer *Eur Urol.* 2003;2(Suppl):3-8.
7. Arnold JT, Isaacs JT. Mechanisms involved in the progression of androgen-independent prostate cancers: it is not only the cancer cell's fault. *Endocr Relat Cancer.* 2002;9:61-73.
8. Bostwick DG. Prostatic intraepithelial neoplasia is a risk factor for cancer. *Semin Urol Oncol.* 1999;17:187-198.
9. Bostwick DG, Neumann R, Qian J, Cheng L. Reversibilty of prostatic intraepithelial neoplasia: implications for chemoprevention. *Eur Urol.* 1999;35(5-6):492-295.
10. Thompson IM, Goodman PJ, Tangen CM, et al. The influence of finasteride on the development of prostate cancer. *N Engl J Med.* 2003;349:213-222.
11. Lazier CB, Thomas LN, Douglas RC, Vessey JP, Rittmaster RS. Dutasteride, the dual 5alpha-reductase inhibitor, inhibits androgen action and promotes cell death in the LNCaP prostate cancer cell line. *Prostate.* 2004;58:130-144.
12. Schmidt LJ, Murillo H, Tindall DJ. Gene expression in prostate cancer cells treated with the dual 5 α-reductase inhibitor dutasteride. *J Androl.* 2004;25:944-953.
13. Veldscholte J, Ris-Staplers C, Kuiper GG, et al. A mutation in the ligand binding domain of the androgen receptor of human LNCaP cells affects steroid binding characteristics and response to anti-androgens. *Biochem Biophys Res Commun.* 1990;173:534-540.
14. Heisler LE, Evangelou A, Lew AM, Trachtenberg J, Elsholtz HP, Brown TJ. Androgen-dependent cell cycle arrest and apoptotic death in PC-3 prostatic cell cultures expressing a full-length human androgen receptor. *Mol Cell Endocrinol.* 1997;126:59-73.
15. Coffey RNT, Watson RWG, Hegarty PK, et al. Priming prostate carcinoma cells for increased apoptosis is associated with up-regulation of the caspases. *Cancer.* 2001;92:2297-2308.
16. McEleny K, Coffey R, Morrissey C, et al. An antisense oligonucleotide to the inhibitor of apoptosis proteins (IAP) sensitises prostate cancer cells to Fas and TNFα-mediated apoptosis. *Prostate.* 2004:59:419-425.
17. Hudson DL, Guy AT, Fry P, O'Hare MJ, Watt FM, Masters JRW. Epithelial cell differentiation pathways in the human prostate: identification of intermediate phenotypes by keratin expression. *J Histochem Cytochem.* 2001;49:271-278.
18. Jiang Z, Woda BA, Rock KL, et al. P504S. A new molecular marker for the detection of prostate carcinoma. *Am J Surg Pathol.* 2001;25:1397-1404.
19. Jiang Z, Fanfer GR, Woda BA, et al. Expression of α-methylacyl-CoA racemase (P504S) in various malignant neoplasms and normal tissues: a study of 761 cases. *Hum Pathol.* 2003; 34:792-796.
20. Kuefer R, Varambally S, Zhou M, et al. α-Methylacyl-CoA racemase: expression levels of this novel cancer biomarker depend on tumour differentiation. *Am J Pathol.* 2002;161: 841-848.
21. Luo J, Zha S, Gage WR, et al. α-Methylacyl-CoA racemase: a new molecular marker for prostate cancer. *Cancer Res.* 2002; 62:2220-2226.
22. Thomas LN, Lazier CB, Gupta R, et al. Differential alterations in 5α-reductase type 1 and type 2 levels during development and progression of prostate cancer. *Prostate.* 2005;63:231-239.
23. Thomas LN, Douglas RC, Vessey JP, et al. 5alpha-reductase type 1 immunostaining is enhanced in some prostate cancers compared with benign prostatic hyperplasia epithelium. *J Urol.* 2003;170:2019-2025.
24. Bayne CW, Ross M, Inglis NF. Induction of 5α-reductase type II mRNA transcription in primary cultured prostate epithelial cells by a soluble factor produced by primary cultured prostate fibroblast cells. *Eur J Cancer.* 2003;39: 1004-1011.
25. Bayne CW, Donnelly F, Chapman K, Bollina P, Buck C, Habib FK. A novel co culture model for benign prostatic hyperplasia expressing both isoforms of 5α-reductase. *J Clin Endocrinol Metab.* 1998;83:206-213.
26. Blanchere M, Berthaut I, Portois MC, Mestayer C, Mowszowicz I. Hormonal regulation of the androgen receptor expression in human prostatic cells in culture. *J Steroid Biochem Mol Biol.* 1998;66(5-6):319-326.
27. Planz B, Tabatabaei S, Kirley SD, et al. Studies on the differentiation pathway and growth characteristics of epithelial culture cells of the human prostate. *Prostate Cancer Prostatic Dis.* 2004;7:73-83.
28. Peehl DM. Primary cell cultures as models of prostate cancer development. *Endocr Relat Cancer.* 2005;12:19-47.
29. Shibata Y, Ito K, Suzuki K, et al. Changes in the endocrine environment of the human prostate transition zone with aging: simultaneous quantitative analysis of prostatic sex steroids and comparison with human prostatic histological composition. *Prostate.* 2000;42:45-55.
30. Ji Q, Liu PI, Elshimali Y, Stolz A. Frequent loss of estrogen and progesterone receptors in human prostatic tumours as determined by quantitative real-time PCR. *Mol Cell Endocrinol.* 2005;229:103-110.
31. Festuccia C, Angelucci A, Gravina GL, Muzi P, Vicentini C, Bologna M. Effects of 5 alpha reductase inhibitors on androgen-dependent human prostatic carcinoma cells. *J Cancer Res Clin Oncol.* 2005;131:243-254.