Androgen induces differentiation of a human papillomavirus 16 E6/E7 immortalized prostate epithelial cell line

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Abstract

Androgen signaling is crucial for the growth and development, as well as for tumorigenesis of the prostate. However, many of the prostate epithelial cell lines developed previously, either normal or tumorigenic, do not express androgen receptor (AR) or respond to androgen. In order to advance our understanding on how androgen signaling regulates the growth and the differentiation status, and affects tumorigenicity of the epithelial cell, we performed experiments on HPr-1, a prostate cell line recently immortalized from normal human prostate epithelial cells. In the present study, AR was stably transfected into

HPr-1 cells by replication-defective retrovirus. Treatment of HPr-1AR cells with androgen resulted in cell differentiation and growth retardation accompanied with up-regulation of cytokeratins K8 and K18, prostate specific antigen, p21 and p27, and down-regulation of c-myc, bcl-2 and telomerase activity. Our results suggest that androgen promotes the process of differentiation in a human papillomavirus 16 E6/E7 immortalized prostate epithelial cell line which may reflect the normal effects of androgen on prostate cells.

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Introduction

Although androgen is known to be important for the growth and development of the prostate, its action is still not fully known. Removal of androgen results in rapid degeneration of the normal and neoplastic prostate cells, as reflected by prostate cancer patients after androgen ablation therapy. Yet among these patients, many of them progress to an androgen independent stage after the therapy, where the tumor starts to re-grow in the absence of androgen (Scott *et al.* 1980). A better understanding of the precise action of androgen on the normal and neoplastic prostate cells is necessary in order to find out how androgen independent prostate cancer may arise and to improve the treatment for prostate cancer.

In the prostate, androgen receptor (AR) is mainly expressed by epithelial and smooth muscle cells (Chodak *et al.* 1992). During the degeneration of the prostate after androgen ablation, epithelial cells are the major cell type that undergo apoptosis (Berges *et al.* 1993), suggesting that the epithelial cell is highly dependent on androgen for survival. Also, the growth of several prostate epithelial cell lines, such as LNCaP (Horszewicz *et al.* 1983), NRP-152 (Danielpour *et al.* 1994) and RWPE-1 (Bello *et al.* 1997), are stimulated by androgen. However, many studies have also shown that the growth of immortalized cell lines or even primary cultures derived from prostate epithelium are not stimulated by androgen (McKeehan *et al.* 1984,

Rundlett *et al.* 1992, Grant *et al.* 1996, Berthon *et al.* 1997). Meanwhile, the study by Cunha & Lung (1978) has demonstrated that urogenital sinus epithelium from testicular feminizing mice, which lacks AR expression, can still develop into a prostate-like structure but only when combined with urogenital sinus mesenchyme that expresses AR. This suggests that androgen acts indirectly through the mesenchyme to stimulate the growth of the epithelial cell. The androgen-signaling cascade in epithelial cells may thus have functions other than growth stimulation.

Previously, Heisler *et al.* (1997) have shown that androgen induces growth arrest and apoptosis in PC-3 cells ectopically transfected with the AR gene. This seems to contradict the generally perceived effect of androgen on prostate epithelial cells. Since PC-3 cells were derived from tumor at an advanced stage, the observation may not reflect the normal effect of androgen on epithelial cells. In this study, we employed a prostate epithelial cell line, HPr-1, for the study of direct androgenic effects on epithelial cells.

The cell line was previously immortalized in our laboratory by the introduction of HPV16 E6/E7 (Choo *et al.* 1999). It was found to be non-tumorigenic but does not express AR and does not respond to androgen stimulation. The reason for the lack of AR expression in HPr-1 cells is uncertain but it is likely to be due to the incomplete microenvironment of the culturing systems. We had the

AR gene expressed in HPr-1 cells by retroviral transduction of a wild type AR cDNA. The resultant cell line, HPr-1AR, was used as a model for the investigation of the effects of androgen on prostate epithelial cells. Since HPr-1AR is non-tumorigenic and morphologically resembles normal prostate epithelial cells, our findings probably reflect the effect of androgen on normal epithelial cells of the prostate.

Materials and Methods

Cell culture

HPr-1 and HPr-1AR cells were maintained in keratinocyte serum free medium (SFM; Gibco, Gaithersburg, MA, USA) supplemented with penicillin G (100 U/ml), streptomycin (100 µg/ml) and gentamicin (50 µg/ml). For experiments, the cells were grown in RPMI medium supplemented with 10% charcoal-dextran-treated fetal bovine serum (charcoal-dextran-treatment removed lipids and lipid soluble steroids including androgen) and various doses (0.1 to 100 nM) of mibolerone (a non-hydrolysable androgen analog) dissolved in ethanol or an equivalent volume of the vehicle. The concentration of mibolerone was found to be optimum at 5 nM and this concentration was used for the rest of the study.

Generation of sub-cell line HPr-1AR

The retroviral vector pBabe-AR was generated by inserting the full-length human AR gene into the pBabe vector (a generous gift from Dr Scott Lowe at Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, USA). Transfection of the packaging cell line PA317 (Miller & Buttimore 1986) with pBabe-AR was carried out with the Fugene 6 transfection reagent (Roche Molecular Biochemicals, Basel, Switzerland), following the protocol provided by the manufacturer. The supernatant of the transfected pBabe-AR PA317 cells was collected 72 h post transfection, and used to infect HPr-1 cells in the presence of 8 µg/ml polybrene. The infected HPr-1 cells were selected with 2 µg/ml puromycin for one week. Positive cells were expanded and further selected with 4 µg/ml puromycin to yield a stably AR transduced HPr-1AR sub-line.

Growth characteristics of HPr-1 and HPr-1AR cell lines

For mibolerone dose-response experiments, HPr-1 or HPr-1AR cells were seeded at 200 000/10 cm², grown in RPMI medium containing 10% charcoal-dextran-stripped fetal bovine serum (FBS) supplemented with either various concentrations of mibolerone or vehicle alone. The cells were harvested and counted after 72 h. For the growth curve experiment, the cells were seeded at

100 000/10 cm² in SFM and switched to RPMI medium supplemented with 10% charcoal-dextran-stripped FBS with 5 nM mibolerone or with vehicle alone 48 h after seeding. At daily intervals (for consecutive 4 days), the cells were trypsinized, washed with PBS, and counted in triplicate with a hemacytometer. Phase contrast microscopy was carried out using a Nikon Phase Contrast-2 microscope at 200 × magnification at the end of the experiment.

Gene expression pattern of HPr-1 and HPr-1AR cells

Test cells were first washed with PBS and then lysed with RIPA buffer (PBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 10 µg/ml phenylmethylsulfonyl fluoride (PMSF), 20 µg/ml aprotinin, 10 µg/ml leupeptin, 100 mM sodium orthovanadate). The cells were collected by a rubber policeman and passed through a 21-gauge syringe. After incubating on ice for 1 h, the sample was centrifuged at 13 000 r.p.m. for 30 min. The protein content of the supernatant was measured using the DC Protein Assay kit (Bio-Rad, Hercules, CA, USA). Protein samples were boiled in 6 × sample buffer (3.5 mM Tris-HCl (pH 6.8), 30% glycerol, 10% SDS, 24% 2-mercaptoethanol, 0.12 mg/ml bromophenol blue). Cell lysate (20 µg) was subjected to SDS-PAGE and transferred to PVDF membranes. The membranes were blocked with 10% skim milk in TBST (20 mM Tris HCl, 137 mM NaCl, 0.1% Tween 20), washed with TBST and probed with mouse monoclonal antibodies against AR, prostate specific antigen (PSA), p21, p27, bcl-2, bax (Pharmingen, San Diego, CA, USA), c-myc, cytokeratin K18 (Santa Cruz, San Diego, CA, USA) or cytokeratin K8 (Sanbio, San Diego, CA, USA). The membranes were washed with TBST and covered with horseradish peroxidase-conjugated secondary antibodies (Amersham International, Amersham, Bucks, UK) at room temperature for 1 h, washed again with TBST and visualized by enhanced chemiluminescence (ECL) according to the manufacturer's recommendations (Amersham International). The secondary antibody was included in the ECL kit. The results of the Western blotting on AR, cytokeratin 8 and 18 were analyzed by densitometer. The presence of PSA was also tested with ELISA according to the manufacturer's protocol (Abbott Laboratories, Abbott Park, IL, USA).

Luciferase assay

Cells (60% confluent) in 25-cm² flasks were transfected with the plasmid pMAMneoLuc (Clontech, Palo Alto, CA, USA) using the Fugene 6 transfection reagent. The pMAMneoLuc contains a luciferase reporter gene under the transcriptional control of the mouse mammary tumor virus (MMTV) promoter, which contains androgen responsive elements. In cells expressing functional AR, treatment with exogenous androgen leads to nuclear

localization of AR and therefore up-regulation of the luciferase gene transcription. After 24 h, the transfected cells were trypsinized and split into two separate 6-well clustered dishes. Mibolerone was added to one group and vehicle alone to the other. Cell lysate was extracted 72 h after transfection and the luciferase activity in the cell lysate was assayed using the luciferase assay kit from Tropix (Bedford, MA, USA), according to the protocol provided by the manufacturer. Each treatment was performed in triplicate.

Expression of the PSA gene

The expression of the PSA gene in HPr-1 and HPr-1AR cells was studied by reverse transcription-polymerase chain reaction (RT-PCR). In brief, HPr-1 and HPr-1AR cells were treated with mibolerone or vehicle for six days. Total RNA was isolated by the Trizol reagent with procedures described by the manufacturer and 1 µg of the total RNA was reverse transcribed with the Expand reverse transcriptase (Roche Molecular Biochemicals) and amplified with a set of PSA specific primers (sense: 5'-GCC TCT CGT GGC AGG GCA GTC-3', antisense: 5'-CAT CAC CTG GCC TGA GGA ATC-3'). An initial denaturation at 95 °C was followed by 30 cycles of PCR (94 °C for 30 s, 68 °C for 1 min), and a final extension at 72 °C for 5 min. The β-actin gene was amplified by a pair of primers (sense: 5'-GTC GGG CGC CCC AGG CAC CA-3', antisense: 5'-CTC CTT AAT GTC ACG CAC GAT TTC-3') to control for the amount of mRNA and for integrity. The PCR conditions are similar to those for PSA. PCR products were electrophoresed in 2% agarose gels, transferred onto Hybond-N membrane (Amersham International) and hybridized with ³²P-end-labeled PSA (5'-AGC TAC CCA CTG CAT CAG GA-3') and β-actin (5'-AAT CCT TCT GAC CCA TGC CCA CCA TCA-3') oligonucleotide probes. The hybridization signal was visualized after autoradiography.

Telomerase activity

Telomerase activity was assayed with the Telomeric Repeat Amplification Protocol (TRAP) (Intergen, Purchase, NY, USA). Briefly, the assay was carried out in a 50 µl reaction with 2 µl cell extract (0.1, 0.3 or 1.0 µg protein), 0.1 mg of each primer (5'-CCC TTA CCC TTA CCC TTA CCC TAA-3' and 5'-AAT CCG TCG AGC AGA GTT-3'), 20 mM Tris-HCl (pH 8.3), 1.5 mM MgCl₂, 63 mM KCl, 0.005% Tween 20, 1.0 mM EGTA, 0.1 mg/ml BSA, 0.25 mM dNTP, 1 U Taq polymerase and 0.4 ml [α -³²P]dCTP (3000 Ci/mmol). PCR amplification was performed for 25 cycles at 94 °C, 50 °C and 72 °C, 30 s each. Cell extracts incubated with RNase for 30 min at 37 °C were included as negative controls.

(A) HPr-1

(B) HPr-1AR

Figure 1 Dose-response assay for HPr-1 and HPr-1AR cells in response to mibolerone. HPr-1 and HPr-1AR cells were treated with vehicle alone or with various concentrations of mibolerone (Mb) and the number of cells was counted after 72 h of treatment. The total cell number is shown as an average of 3 replicates. Proliferation of HPr-1AR cells started to decline at 0.1 nM Mb and the maximum effect was observed at 5 nM Mb. Proliferation of HPr-1 cells was not affected by Mb at the concentrations used.

Results

Growth characteristics of the HPr-1 and HPr-1AR cell lines

Proliferation of HPr-1 and HPr-1AR cells was tested with various concentrations of mibolerone for a period of 3 days (Fig. 1). Minor attenuation (~20%) of the proliferation of HPr-1AR cells was seen with 0.5 nM mibolerone, and the effect was most pronounced at 5 nM. No further growth inhibition was observed with mibolerone concentrations higher than 5 nM. Therefore, 5 nM mibolerone were used in the rest of this study. Growth of HPr-1 cells was not affected by the presence of mibolerone at the dose range tested, suggesting that the cells did not respond to mibolerone treatment. In time-course experiments (Fig. 2), inhibition of proliferation of HPr-1AR cells by mibolerone was evident at 48 h and most cells were growth arrested at 72 h. Growth regulation of HPr-1AR by mibolerone was completely abolished by pretreating the cells with 10 µM hydroxyflutamide (an androgen antagonist) for 20 h before the addition of mibolerone.

Figure 2 Proliferation of HPr-1 and HPr-1AR cells in response to mibolerone. HPr-1 and HPr-1AR cells were treated with either mibolerone (MB; 5 nM) or vehicle and the number of cells was counted every 24 h. The total cell number is shown at each time interval. The growth rate of HPr-1AR cells declined 48 h after androgen treatment, while HPr-1AR cells grown in the absence of MB and HPr-1 cells (irrespective of the addition of MB) continued to proliferate throughout the experiment.

(A)

(B)

(C)

Figure 3 Cell morphology under phase contrast microscopy. HPr-1AR cells treated with ethanol (A) or androgen (B) for 72 h. (A) HPr-1AR cells treated with ethanol grew to 100% confluence at 72 h. (B) In the presence of androgen, HPr-1AR cells demonstrated differentiated morphology and displayed growth retardation. Androgen had no effect on the morphology of HPr-1 cells (data not shown). In addition, the morphological changes induced by mibolerone in HPr-1AR cells were reversed by the addition of 10 mM hydroxyflutamide (C).

Figure 3 shows the morphology of HPr-1 and HPr-1AR cells in the presence and absence of mibolerone. As indicated in the figure, mibolerone treatment induced striking morphological changes in HPr-1AR. The morphology of HPr-1AR cells became differentiated when compared with the untreated HPr-1AR cells and persisted as colonies which did not grow to confluence at the time when the untreated cells reached 100% confluence. This phenomenon was absent in HPr-1 cells treated with mibolerone or in mibolerone-treated HPr-1AR cells blocked with 10 mM hydroxyflutamide, suggesting that the morphological alterations and growth inhibition of HPr-1AR cells were induced by the androgen-signaling cascade.

Expression of functional AR in HPr-1 and HPr-1AR cells

In order to verify that the differential effects of mibolerone on HPr-1 and HPr-1AR cell lines were due to expression of functional AR, Western blot of AR was performed. No AR was detected in the parental HPr-1 cell line, but in the HPr-1AR sub-cell line a 110 kDa band was visualized by Western blotting of AR (Fig. 4). LNCaP, a lymph node metastatic prostate cell line expressing AR, was used as a positive control for the blotting. The level of AR in HPr-1AR cells was about threefold higher than in LNCaP cells. In functional assays of the AR with pMAMneoLuc plasmid, mibolerone induced a 15-fold increase in luciferase activity

Figure 4 Western blot analysis of AR expression in LNCaP, HPr-1AR and HPr-1 cells. Twenty milligrams whole cell lysate were loaded for each sample. LNCaP, an AR positive prostate cell line was included as control. HPr-1AR cells expressed AR at a level that was 300% higher than LNCaP cells. HPr-1 cells were negative for AR expression.

in HPr-1AR cells, confirming that the expressed AR was functional. The parental HPr-1 cells were not responsive to mibolerone (Fig. 5).

Expression pattern of the differentiation-related genes in HPr-1 and HPr-1AR cells

To determine if the growth arrest observed in HPr-1AR cells after mibolerone treatment was due to induction of cellular differentiation, the expression of cytokeratins K8 and K18, which are up-regulated in prostate epithelial cells during differentiation (Sherwood *et al.* 1989, 1991), was studied by Western analysis. Immunoreactive bands representing the cytokeratins K8 and K18 respectively, were detected in both HPr-1 and HPr-1AR cells (Fig. 6). After mibolerone treatment, the protein levels of cytokeratins K8 and K18 in HPr-1AR cells were found to have increased by around 6- and 8-fold respectively when compared with the expression in untreated cells, while the cytokeratin expression of HPr-1 cells was unchanged following mibolerone treatment.

In addition, the expression of p21 and p27, which are also up-regulated in differentiating epithelial cells (Tian & Quaroni 1999), was also found to be increased in HPr-1AR cells treated with mibolerone. The level of both proteins increased substantially after 24 h of androgen treatment and the level persisted throughout the next 48 h. Finally, c-myc and bcl-2, which are down-regulated in terminal differentiated cells (Grolli *et al.* 1997), were also shown to decrease after mibolerone treatment in HPr-1AR cells (Fig. 7). As shown in the figure, the down-regulation of c-myc was rapid and drastic, while an observable decrease in bcl-2 could only be found after 72 h of androgen treatment. The expression of bax was not affected by androgen in either HPr-1 and HPr-1AR cells.

PSA is an important marker for prostate cells and is highly expressed in terminally differentiated epithelial cells

Figure 5 Luciferase activity assay. HPr-1 and HPr-1AR cells transfected with pMAMneoLuc plasmid were treated with 5 nM mibolerone (Mb) in ethanol or with ethanol alone. Cells were then lysed and assayed for luciferase activity. Results are represented as the level of luciferase induction by androgen compared with normal (100%). Androgen increased luciferase activity (greater than 15-fold) in HPr-1AR cells but had no effect in HPr-1 cells.

(Coffey 1993). However, we were unable to detect expression of PSA in HPr-1 or HPr-1AR cells. To determine if the absence of PSA expression was due to inactive transcription or transcript translation, we looked for PSA mRNA by RT-PCR. In HPr-1AR cells, PSA mRNA was not detected after the first day of androgen treatment. After three days of treatment, PSA mRNA started to appear and almost doubled its level at day six of treatment (Fig. 8). No PSA mRNA could be detected in HPr-1 after six days of androgen treatment or in HPr-1AR treated with vehicle for six days. The results indicated that PSA transcript was induced in HPr-1AR cells by androgen.

Telomerase activities

Decrease in telomerase activity upon induction of differentiation has been reported in a number of immortalized

Figure 6 Expression of cytokeratins (C) K8 and K18. Total cell lysates of HPr-1AR cells treated with mibolerone (Mb) or ethanol for 6 days were subjected to Western blot analysis for protein levels of CK8 or CK18. β -Actin was added as loading control (not shown). Both of the differentiation-specific cytokeratins were found to be up-regulated after the treatment.

cell lines (Sharma *et al.* 1995), as well as in some carcinoma cell lines (Savoysky *et al.* 1996). To further demonstrate that HPr-1AR cells were undergoing differentiation, telomerase activity in HPr-1AR cells grown in the presence or absence of androgen was determined by the TRAP assay. As demonstrated in Fig. 9, telomerase activity of HPr-1AR cells was unchanged after six days of mibolerone treatment, but was then sharply down-regulated at day 9 of the treatment. In non-treated HPr-1AR cells, telomerase activity remained unchanged.

Discussion

As described previously, the HPr-1 cells we used in our study neither express AR nor respond to androgen. Similarly, several of the established prostate epithelial cell lines, such as BPH-1 (Hayward *et al.* 1995), PC-3 (Kaighn *et al.* 1979) or DU145 (Mickey *et al.* 1980) were also found to be AR negative. Previously, Bello *et al.* (1997) had established a human prostate epithelial cell, RWPE-1, which represented one of the few immortalized non-

Figure 8 RT-PCR for PSA. RNA was extracted from HPr-1 or HPr-1AR cells treated with 5 nM mibolerone (Mb) or vehicle. RNA of LNCaP (shown as the last lane) served as a positive control. One microgram of each sample was subjected to RT-PCR for the transcripts of PSA or β -actin. The RT-PCR products were probed with 32 P-labeled oligo-nucleotides specific for PSA or β -actin. Expression of the PSA transcript showed a marked increase after androgen treatment.

tumorigenic cell lines showing AR expression and growth stimulation by androgen. Since HPr-1 and RWPE-1 were both immortalized by human papilloma virus, the difference in AR expression and androgen responsiveness is unlikely to be due to the variation in immortalization method. Meanwhile, the results from RT-PCR and Western blotting of AR (data not shown) have shown that AR was absent in both the parental and HPr-1 cells, suggesting that the lack of AR expression did not result from immortalization. The study by Blanchere *et al.* (1998) demonstrated that stromal-epithelial interaction is important for optimal expression of AR. The lack of stromal-epithelial interaction in culture systems may partly explain the absence of AR in HPr-1 cells. Meanwhile, it is known that AR are mainly expressed in differentiated prostate epithelial cells (Prins *et al.* 1991). The prolonged passage of the parental cell, which occurred before the immortalization of the HPr-1 cells, may lead to the loss of the more

Figure 7 Expression levels of p21, p27, bcl-2, bax and c-myc were modulated by androgen treatment in HPr-1AR cells. Total cell lysates of HPr-1AR cells were obtained after 24, 48 and 96 h mibolerone (Mb) treatment. An equivalent amount of vehicle was added to the negative controls. Western blot analyses revealed that bcl-2 and c-myc were down-regulated, while p21 and p27 were up-regulated in HPr-1AR cells during androgen treatment. The level of bax remained unchanged during the treatment.

Figure 9 TRAP assay for telomerase activities. Aliquots of each cell extract (0.1, 0.3 and 1 µg) were used for the reaction. A decrease in telomerase activity was observed after 9 days of androgen treatment (mibolerone, Mb). Water (H₂O) and boiled cell extract (Δ) served as negative controls.

differentiated AR positive cell, leaving only the less differentiated AR negative cell for immortalization.

Similar to that which Heisler *et al.* (1997) have shown, expression of the wild-type AR in HPr-1 cells resulted in growth retardation under androgen treatment. However, the androgen-induced apoptosis, which has been observed in PC-3 cells, was not detected in androgen-treated HPr-1AR cells, either by flow cytometry or DNA laddering (data not shown). Apoptosis is known to correlate with differentiation, since cells incapable of differentiation always go through programmed cell death when exposed to differentiation stimulus (Martin *et al.* 1990, Atencia *et al.* 1994). HPr-1AR cells differ from PC-3 cells in their non-tumorigenic and apparently normal phenotype. The knowledge that androgen is important for the differentiation of the prostate led us to speculate that androgen might act directly on HPr-1AR cells to stimulate their differentiation, and the induction of apoptosis by androgen on AR-expressing PC-3 cells may be due to the loss of their ability to differentiate upon androgen stimulation. Indeed, the observed changes in HPr-1AR cells treated with androgen agree with our hypothesis.

Morphologically, HPr-1AR cells treated with androgen appeared to undergo differentiation as they became flattened and enlarged, thus resembling epithelial cells undergoing differentiation. Androgen also modulated the

expression of cytokeratins and PSA in HPr-1AR cells. The profile of cytokeratin expression is a useful biochemical marker to determine the origin and differentiation status of cells (Moll *et al.* 1982, Martin *et al.* 1990). In particular, cytokeratins K8 and K18 have been regarded as tissue-specific and as differentiation markers in prostate epithelial cells (Sherwood *et al.* 1989, 1991). PSA, on the other hand, is another important biochemical marker of prostate epithelial cells (Sinha *et al.* 1986, Fong *et al.* 1991). It is expressed mainly in differentiated secretory cells and is androgen dependent (Wolf *et al.* 1992, Savoysky *et al.* 1996). The up-regulation of cytokeratins K8 and K18, together with the induction of PSA transcript in HPr-1AR cells during androgen treatment provided strong evidence that the cells were undergoing differentiation under the influence of androgen. Although the result from Southern blotting showed that the mRNA level of PSA in HPr-1AR after six days of androgen treatment is comparable to that of LNCaP, we were unable to detect PSA protein by Western analysis and ELISA with a few of the commercially available PSA antibodies (data not shown). Our suggestion is that the absence of PSA expression is probably due to the incomplete microenvironment in cell culture which is unfavorable to the post-transcriptional processing of PSA.

Cellular differentiation involves the interplay of a concert of gene expression. In our study, we have monitored the expression of p21, p27, c-myc and bcl-2 proteins in response to androgen treatment in HPr-1AR cells. The p21 and p27 proteins are cyclin-dependent kinase inhibitors (CKI) that are involved in the control of the cell cycle, and their induction has been correlated with cell growth arrest and differentiation of various cell lines (Tian & Quaroni 1999). Western blot analysis indicated that the expression of both p21 and p27 in HPr-1AR cells was modulated by androgen. However, it should be noted that the level of p21 and p27 detected in HPr-1 or the vehicle-treated HPr-1AR cells also increases progressively, presumably due to the increase in contact inhibition as the cells were becoming confluent. Since the androgen-treated HPr-1AR cells remained half confluent at 72 h, the increase in p21 and p27 expression was likely due only to the effect of androgen. A recent study of the promoter of the p21 gene has identified the presence of an androgen responsive element in the promoter region of the p21 gene (Lu *et al.* 1999), suggesting that p21 may be involved in androgen signaling. It is thus possible that the up-regulation of p21 in HPr-1AR cells is due to transactivation of the p21 gene by androgen.

The c-myc gene is a proto-oncogene encoding a transcription factor (Grolli *et al.* 1997) and bcl-2 functions as an inhibitor of apoptosis (Lu *et al.* 1996). While both c-myc and bcl-2 were down-regulated by androgen in HPr-1AR cells, the drop in c-myc levels was much more drastic. A sharp decline of c-myc levels has also been

observed in LNCaP cells that have been induced to differentiate by phorbol 12-myristate 13-acetate (PMA) (Blagosklonny *et al.* 1997), suggesting that the observed decrease in c-myc expression in HPr-1AR cells might be due to cell differentiation during androgen treatment. The down-regulation of bcl-2 in HPr-1AR cells was less obvious, which might in fact explain the absence of apoptosis following androgen treatment.

Finally, telomerase activity was also found to be modulated in HPr-1AR cells under androgen treatment. Telomerase is a ribonuclear protein that functions as the enzyme for the addition of tandem repeats (TTAGGG)_n at the end of chromosomes (Morin 1989). Most of the immortalized cell lines and germ cells are found to be positive for telomerase activity (Kim *et al.* 1994, Lu *et al.* 1996). Conversely, telomerase activity has been reported to be absent in terminally differentiated cells (Sharma *et al.* 1995, Bestilny *et al.* 1996). We showed that androgen treatment down-regulated telomerase activity in HPr-1AR cells, which was consistent with differentiation of the cells. However, down-regulation of the telomerase activities started only after 9 days of androgen treatment, long after the occurrence of growth arrest and morphological changes. This could possibly be explained by the differentiation status of HPr-1AR cells. In other words, HPr-1AR cells treated with androgen for nine days might have a higher degree of differentiation, so that suppression of telomerase was observed.

It is commonly believed that androgen is essential for the proliferation of prostate epithelial cells, as supported by a number of *in vitro* models such as LNCaP (Horszewicz *et al.* 1983) or RWPE-1 (Danielpour *et al.* 1994). In particular, LNCaP cells also show a biphasic response towards androgen (Sonnenschein *et al.* 1989), with growth stimulated at low concentrations but inhibited at high concentrations of androgen. Interestingly, Geck *et al.* (1999) have isolated two sub-populations of LNCaP cells that express different levels of AR and respond differently to androgen. For LNCaP cells that express high levels of AR, androgen will induce growth arrest, while in cells that have low levels of AR expression, androgen will stimulate cell growth. Since the AR gene in LNCaP is mutated, its response to androgen is unpredictable and can be stimulated by androgen antagonists and other steroids (Veldscholte *et al.* 1992); it is uncertain if the observed response to androgen in LNCaP also holds true for normal prostate epithelial cells. This highlights the need for a well-characterized and homogenous prostate cell line to properly address the effect of androgen in prostate epithelial cells. The HPr-1AR cell line generated in our laboratory is a well-characterized androgen-responsive non-tumorigenic cell line. We believe that this cell line provides a good model to study androgen effects on normal prostate cell biology. Further genetic manipulation of this cell line may allow for the generation of a step-wise model of prostate cancer development and provide future oppor-

tunities to study the effects of androgen on tumorigenic prostate cells.

Androgen ablation is still the first line treatment for prostate cancers, although many patients will eventually suffer from a progression to an androgen independent stage (Scott *et al.* 1980). Improvements in the current treatment for prostate cancer are urgently needed as the incidence of prostate cancer keeps rising every year and worldwide (Rise *et al.* 1997). Currently, an intermittent androgen ablation treatment is under clinical trial which, as reported by some of the study groups (Oliver *et al.* 1997, Rambeaud 1999) has shown beneficial effects to patients by delaying the emergence of hormone independent cancer. It is believed that the withdrawal of androgen blockage at intervals may allow the cancer cells to retain certain degrees of differentiation as well as androgen dependency (Sato *et al.* 1996). Our finding that androgen can stimulate prostate epithelial cells to differentiate *in vitro* may form the theoretical basis for the development of new therapies for prostate cancer.

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