Increased Androgen Receptor Activity and Altered c-myc Expression in Prostate Cancer Cells after Long-Term Androgen Deprivation

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ABSTRACT

Proliferation of LNCaP 104-S cells, a clonal subline of the human prostate cancer cell line, was very slow in androgen-depleted medium but increased 10-13-fold in the presence of 0.1 nM of a synthetic androgen, R1881. This induction of proliferation was diminished at higher concentrations of R1881, indicating the biphasic nature of the androgen effect. After 20-30 passages in androgen-depleted medium, these cells progressed to 104-I cells, which exhibited much lower proliferative sensitivity to 0.1 nM R1881. After another 20-30 passages, LNCaP 104-I cells gave rise to 104-R cells, which proliferated rapidly without additional androgen. Proliferation of 104-R cells was induced 2-fold by 0.01 nM R1881 but was repressed by 0.1 nM R1881 and above. Thus, androgen induction and repression of proliferation could be seen at lower concentrations of androgen as the cells progressed. During the transition of 104-S cells to 104-R cells, the androgen receptor mRNA level increased 2.5-fold whereas the androgen receptor protein level increased 15-fold in the absence of androgen. Androgen receptor transcriptional activity, measured by androgen induction of prostate-specific antigen mRNA and chromomethylphosphinemethylcyltransferase activity in transfected cells, increased up to 20-fold during the progression. LNCaP cells, therefore, appear to be able to adapt to reduced androgen availability by increasing their sensitivity to androgen, raising questions concerning the therapeutic strategies used against prostate cancer. Androgen induction of c-myc expression in 104-R cells occurred at a 10-fold lower concentration (0.01 nM) than in 104-S cells (0.1 nM). In all stages, cell proliferation and c-myc expression were repressed by androgen at a high concentration (20 nM), but the repression of cell proliferation was blocked by retroviral overexpression of c-myc.

INTRODUCTION

Prostate cancer is currently the most common malignancy and is the second leading cause of cancer death among males in the United States (1). The mechanism of prostatic carcinogenesis and tumorgenesis likely involves a multistep progression from precancerous cells to cells which proliferate and metastasize. The growth and development of prostate cancer appears to be androgen dependent initially, making it vulnerable to androgen ablation and antiandrogen therapies (2). Most often, however, prostate cancer cells lose androgen dependency and responsiveness during the progression to malignant stages, and tumor cells which are resistant to endocrine therapy ultimately proliferate. While loss of AR expression may accompany loss of androgen dependency and responsiveness (3), other prerequisite cellular events probably occur which allow cell proliferation to bypass the androgen requirement. Loss of AR expression may, therefore, either drive the selection for such events or occur secondarily (4).

The androgen-responsive human prostatic carcinoma cell line LNCaP (5, 6) has been used extensively as an in vitro model for examining the role of the AR in the control of prostatic carcinoma cell proliferation. LNCaP cells express large amounts of AR mRNA and protein. The levels of AR mRNA but not protein appear to be under moderate negative control by androgen (7-9). ARs in LNCaP cells possess a mutation in the androgen binding domain which alters the specificity of ligand binding and steroid-induced transactivation (10, 11) and is probably responsible for the aberrant proliferative response of LNCaP cells to antiandrogens (12-14).

We were interested in determining whether changes might occur in the proliferative response of LNCaP cells to androgen when cells are cultured over a long period of time in androgen-depleted medium and if so, to identify the accompanying molecular changes. If androgen sensitivity could be altered in a clonal isolate of androgen-sensitive LNCaP cells by long-term androgen deprivation, it should be helpful in the study of molecular and/or cytogenetic changes which occur concomitantly with the observed changes in proliferative response. A demonstration that changes in specific gene expression take place over time in a clonal isolate would support the idea that prostate tumor cells are able to adapt to lowered androgen concentration in their environment and that androgen-independent cells do not necessarily arise during androgen ablation therapy through selection of a preexisting, androgen-independent subpopulation (15). The use of a clonal subline also simplifies the interpretation of androgen response in a heterogeneous parental cell population. We were also particularly interested in changes in the expression of c-myc, because expression of this critical growth-related protooncogene has been reported to be negatively regulated by high concentrations of androgen and positively correlated with proliferation in LNCaP cells (16, 17). Deregulated expression of c-myc has been observed in a variety of neoplasias and has been implicated in neoplastic development (for reviews, see Refs. 18-21). Elevated c-myc expression has been observed in benign prostatic hyperplasia and prostatic carcinoma (22, 23). In addition to having a role in the induction of cell proliferation, c-myc also appears to function under certain circumstances (overexpression during growth factor withdrawal) in apoptosis (24-27).

In this paper, we report on the results of experiments with an androgen-responsive LNCaP 104 clonal subline subcultured in androgen-depleted medium for about 60 passages. As the 104 cells progressed, they became more sensitive to low concentrations of androgen, and proliferative response of this subline to R1881 at 0.1 nM concentration changed from positive to negative. LNCaP 104 cells appeared to adapt to lowered androgen levels by increasing AR expression and transcriptional activity. These results show that androgen regulation of c-myc expression may play an important role in the apparent androgen independence of prostate cancer cells. These findings additionally suggest that in designing a therapeutic approach to the later stages of prostate cancer that show apparent loss in androgen-dependent proliferation, it is necessary to consider the underlying mechanism and the usefulness of hormonal intervention.
MATERIALS AND METHODS

Materials. The LNCaP cell line was obtained from Dr. T. M. Chu at the Roswell Park Memorial Institute (Buffalo, NY), and the androgen-insensitive prostate cancer PC-3 cell line was obtained from American Type Tissue Collection. [α-32P]dCTP (3000 Ci/mmol), [α-32P]UTP (800 Ci/mmol), and the Multiprime Random Primer Kit were purchased from Amersham. Restriction endonucleases and other enzymes were purchased from Boehringer Mannheim, Stratagene, BRL, and Promega. AN-21, a polyclonal rabbit antibody raised against a 21-amino acid peptide corresponding to the NH2-terminus of human and rat AR (28, 29) was prepared in this laboratory by Drs. Ching Song and Richard Hiipakka using methods described by Tsang and Wilkins (30). An enhanced chemiluminescence detection kit was purchased from Amersham. A luciferase assay system kit was purchased from Promega. R1881 was from New England Nuclear, and other steroids were from Steraloids. The MMTV-CAT vector was derived from pMSG-CAT (Pharmacia) by the excision of the 2-kilobase BamHI fragment containing the gpt gene (11).

LNCaP Subline Isolation, Cell Culture, and Retroviral Infection. The LNCaP 104-S subline was isolated with 11 other sublines by cloning individual colonies after seeding serially diluted LNCaP cells. LNCaP sublines were then maintained in DMEM (Gibco) supplemented with 1 nM DHT and 10% FBS (Upstate Biotechnology, Inc.). LNCaP 104-I cells were derived from the LNCaP 104-S subline by continuous passage, starting at passage 40 in DMEM supplemented with 10% CS-FBS (31), and were subsequently maintained in the same medium (Fig. 1A). As an assay of residual androgenic activity in CS-FBS, CAT activity in PC-3 cells incubated in 10% CS-FBS and transiently transfected with MMTV-CAT and pSG5-LNCaP AR (11) expression vectors was about 20% of the level in PC-3 cells treated with 0.1 nM R1881. By the same assay, androgenic activity present in medium supplemented with 10% untreated FBS was equivalent to the activity of 0.05 nM R1881. LNCaP 104-R cells derived from 104-I cells were also maintained in DMEM supplemented with 10% CS-FBS. One passage consisted of 7 days of growth to confluency, starting from a 1:9 dilution of a trypsinized cell suspension. Responsiveness of cell proliferation to various concentrations of R1881 was measured by determining the cell density of trypsinized cell suspensions with a hemocytometer after 6 days of growth. Cells were plated in triplicate in 12-well dishes (3 × 104 cells/well) in DMEM supplemented with 10% CS-FBS in the presence or absence of R1881. In instances of cell clumping, pelleted cells were treated with a solution of 0.4% Nonidet P-40, 50 mM Tris, and 5 mM MgCl2, pH 7.5, and the density of free nuclei was determined with a hemocytometer. LNCaP 104 sublines were infected with retrovirus generated from the pMV7 retroviral vector (32) using the procedures of Brown and Scott (33). The pMV7 vector contains the gene encoding neomycin phosphotransferase, which confers selectable resistance to geneticin (G418; Gibco). A 1.5-kilobase EcoRI-HindIII human c-myc cDNA fragment was inserted into EcoRI-HindIII-digested pMV7, which was transfected into the mouse pJ2 packaging cell line using the calcium phosphate precipitation technique. After 2 weeks of selection in medium containing 4 mg/ml geneticin, ectopic viral progeny produced from geneticin-resistant cells were used to infect mouse PA317 cells, which produced amphotrophic retrovirus capable of infecting human cells. Retrovirally infected LNCaP cells were maintained in DMEM supplemented with 10% untreated FBS and 0.5 mg/ml geneticin.

Anchorage-independent Growth in Agarose. Low melting point agarose (FMC Bioproducts) was suspended at 0.75% in water, autoclaved, and cooled to 50°C. One-tenth volume of 10 × DMEM and 0.1 volume of either CS-FBS or untreated FBS were added to make a final agarose concentration of 0.6%. Three ml was dispensed into 6-cm tissue culture plates. LNCaP cells (2 × 105) were suspended in 1 ml of DMEM supplemented with either 10% CS-FBS or 10% FBS and with R1881 at a concentration 2-fold higher than the final one. One ml of the 0.6% agarose-DMEM-FBS solution was added to the cells, and the cell suspension was poured over the previously gelled agarose layers. After 13 days, colonies on duplicate plates were randomly scored as less than or greater than 200 μm in diameter along the largest dimension until 100 colonies in either size category were counted.

RNA Analysis. Cells were grown in the presence or absence of R1881 to 80% confluency (4–5 days). Four h prior to harvesting, culture medium was replaced with fresh medium and R1881. Total RNA was isolated from scraped cells using the guanidinium thiocyanate lysis-LiCl precipitation technique (34). Ribonuclease protection assay was performed as described by Zinn et al. (35) and Hay et al. (36). Samples (20 μg of total RNA plus 32P-labeled RNA probes) were denatured at 80°C for 7 min and were hybridized at 56°C for 14–16 h. RNase treatment was with 35 μg/ml RNase A and 1000 units/ml T1 (Sigma) at 31°C for 50 min, followed by the addition of 125 μg/ml proteinase K (Merck) for 10 min at 37°C. After phenol-chloroform extraction and ethanol precipitation, RNase-resistant fragments were analyzed by autoradiography and quantitation with an AMBIS radiobioanalytic imaging system (AMBIS Systems, San Diego, CA). For detection of AR mRNA, a 210-base pair KpnI-SacI fragment spanning a portion of the DNA-binding domain (28) was inserted into pBlueScript SK+ for in vitro transcription of 32P-labeled antisense RNA with T3 RNA polymerase. For detection of PSA mRNA, a 77-base pair fragment specific to PSA was generated by polymerase chain reaction amplification of a 2-μl aliquot of a cDNA library using the two 22-base primers, 5′-AGCCTA-GAGAAGGCTGTCAGCC-3′ and 5′-AGGCCCTCTCTTATTACATCC-3′ (37). The 77-base pair fragment was inserted into the EcoRV site of pBlueScript SK+ for transcription of 32P-labeled antisense RNA with T3 RNA polymerase. The PSA sequence and fragment orientation were confirmed by dideoxy sequence analysis. For detection of c-myc mRNA, a 1.5-kilobase EcoRI-HindIII cDNA fragment inserted into pGEM-3 was used for transcription of 32P-labeled antisense RNA with SP6 RNA polymerase (38). A 600-base pair probe was generated by in vitro transcription with primer 5′-CCATTACCGGATCCTGGCGGGA CATG-3′ (37). A 144-base pair probe was generated by in vitro transcription and dried down to about 200 pmol for use with in situ hybridization techniques. The cell suspension was poured over the previously gelled agarose layers. After 13 days, colonies on duplicate plates were randomly scored as less than or greater than 200 μm in diameter along the largest dimension until 100 colonies in either size category were counted.

Fig. 1. A, strategy of androgen deprivation in LNCaP 104 cells. Starting at passage 40, LNCaP 104-S cells were grown in DMEM supplemented with 10% CS-FBS. LNCaP 104-I and LNCaP 104-R cells arose at the indicated passage numbers. B, dose-response curve of LNCaP 104 cell proliferation over a range of R1881 concentration. Cells (3 × 104/well) were plated in triplicate wells in a 12-well multiwell plate and grown in DMEM supplemented with 10% CS-FBS and R1881 at the indicated concentrations. Cell densities were determined after 6 days of growth. LNCaP 104-S, 104-I, and 104-R cells were tested at passages 16, 65, and 95, respectively.
Fig. 2. Effect of 0.1 nM R1881 on anchorage-independent growth of LNCaP 104 cells in agarose. The percentage of colonies greater than 200 μm in diameter was determined after 13 days of growth in DMEM supplemented with either 10% CS-FBS or 10% untreated FBS in the presence or absence of 0.1 nM R1881.

AR Immunoblotting. LNCaP 104-S, LNCaP 104-I, 104-R, and PC-3 cells were grown in the presence or absence of R1881 for 4 days in DMEM supplemented with 10% CS-FBS. Cells were washed in PBS, trypsinized, and resuspended in DMEM supplemented with 10% CS-FBS. After counting, aliquots containing 10⁶ cells were centrifuged. Cell pellets were resuspended in 100 μl of 2X SDS-PAGE loading buffer and heated at 95°C for 5 min. Proteins present in 10-μl aliquots (10⁵ cells) were separated on 10% SDS-PAGE gels and electroblotted onto nitrocellulose filters. Nonfat dry milk-blocked filters and electroblotted onto nitrocellulose filters. Nonfat dry milk-blocked filters were successively incubated with 0.1 μg/ml affinity-purified AN-21 polyclonal rabbit anti-AR antibody, and a 1:2000 dilution of goat anti-rabbit IgG conjugated with horseradish peroxidase (Amersham) in PBS containing 0.2% Tween 20 and 5% nonfat dry milk. Each incubation was for 1 h at room temperature. Unbound antibody was removed after each incubation by washing for 1 h (four changes) in PBS with 0.2% Tween 20 at room temperature. The enhanced chemiluminescence reagent was prepared and used according to the instructions of the supplier. The relative amount of AR staining was quantified by densitometric scanning of film after a 10-s exposure to filters immediately after the chemiluminescence reaction was initiated.

MMTV-CAT Transactivation. The MMTV-CAT vector (20 μg) containing the MMTV-LTR linked with the CAT gene was cotransfected with a pRSVL vector (2 μg) into LNCaP 104-S, LNCaP 104-I, and LNCaP 104-R cells on 10-cm plates (5 plates/treatment) using the calcium phosphate precipitation method. The pRSVL vector contains the luciferase gene linked to the RSV LTR (40). Cells, incubated in DMEM supplemented with 10% CS-FBS, were treated with 1 nM R1881 or ethanol at the time of transfection. Cells were harvested 60 h later. Cell extracts were isolated using reporter lysis buffer (25 mM bicine-0.05% Tween 20-0.05% Tween 80, pH 7.6; Promega), and luciferase activity was assayed according to the procedure supplied by Promega. Cell extracts were normalized within cell line groups for luciferase activity, and aliquots containing equivalent luciferase activity were used in 4-h CAT reactions at 37°C. Acetylation of [14C]chloramphenicol was quantified after thin layer chromatography using the AMBIS radioanalytic imaging system.

RESULTS

Isolation of 104-I and 104-R Cell Lines Exhbiting Altered Proliferative Response to Androgen. Cells making up the LNCaP prostate carcinoma cell line population display a heterogenous proliferative response to androgen, indicative of variable changes taking place within the population in the molecular mechanisms mediating the hormonal response. In order to identify molecular processes which accompany the proliferative changes, the proliferative behavior of one LNCaP clonal isolate grown under steroid-depleted conditions was examined over time (Fig. 1B). This subline, designated 104-S, exhibited the strongest proliferative stimulation by androgen when the cells were grown in medium supplemented with CS-FBS; 0.1 nM R1881 stimulated proliferation of 104-S cells by 10- to 13-fold. When LNCaP 104-S cells were subcultured in medium supple-
AR mRNA levels in 104-R cells were 2.5-fold higher than the levels detected in LNCaP 104-S cells grown in the absence of androgen (Fig. 3). Incubation of 104-S cells in increasing concentrations of R1881 resulted in a gradual reduction of AR mRNA level, while incubation of 104-R cells in 0.1 nM R1881 caused a sharp drop in AR mRNA level when the level of AR mRNA was normalized to the level of β2-MG mRNA. AR mRNA was not detected in the androgen-insensitive PC-3 cells (data not shown).

Immunoblot analysis of AR content in LNCaP whole cell extracts normalized by cell number showed that the level of the M, 100,000 AR peptide in LNCaP 104-I and 104-R cells grown in medium with androgen-depleted CS-FBS was about 3-fold and 15-fold higher, respectively, than that of 104-S cells (Fig. 4). Growth of 104-S cells over a range of R1881 concentration resulted in a dose-dependent increase of AR protein content with maximal enhancement of nearly 5-fold observed with 10 nM R1881. This induction of AR protein level by androgen in 104-S cells is consistent with findings of Krongrad et al. (8), but the scale of the induction in 104-S cells is much greater. R1881 treatment of 104-I cells produced a maximal 2- to 3-fold increase. Treatment of 104-R cells with R1881, on the other hand, resulted in no increase over the already high basal level when 0.01 and 0.1 nM R1881 were used and reduced AR levels by 50% when 1 and

DMNT > 5α-DHT >> progesterone > 17β-estradiol, whereas dexamethasone had no effect.

The proliferative response of LNCaP 104-S, 104-I, and 104-R cells to 0.1 nM R1881 was also examined by measuring anchorage-independent colony growth in agarose in the presence of CS-FBS or untreated FBS (Fig. 2). While 0.1 nM R1881 was essential for significant growth of 104-S colonies in 10% CS-FBS, R1881 increased only by about 75% the growth of 104-I colonies and almost completely inhibited the growth of 104-R colonies. In the presence of untreated FBS that obviously contained endogenous androgens, addition of R1881 to media did not affect the growth of LNCaP colonies at any stage. Endogenous androgens may have been sufficient for maximal stimulation of the proliferation of 104-S and 104-I cells and inhibition of 104-R cell proliferation.

**AR Expression in LNCaP 104 Cell Lines.** To determine whether changes in AR expression and activity were concomitant with changes in the proliferative response of LNCaP 104 cells to androgen, AR mRNA level, protein level, and androgen induction of PSA mRNA level and MMTV promoter activity were measured. Northern blot analysis of total RNA from LNCaP 104-S and 104-R revealed the presence of the major form of AR mRNA, approximately 10 kilobases in length (data not shown). For more sensitive measurement of AR mRNA levels, RNase protection assay was performed using a 210-base antisense RNA probe. When normalized to the amount of β2-MG mRNA, AR mRNA levels in 104-R cells were 2.5-fold higher than the levels detected in LNCaP 104-S cells grown in the absence of androgen (Fig. 3). Incubation of 104-S cells in increasing concentrations of R1881 resulted in a gradual reduction of AR mRNA level, while incubation of 104-R cells in 0.1 nM R1881 caused a sharp drop in AR mRNA level when the level of AR mRNA was normalized to the level of β2-MG mRNA. AR mRNA was not detected in the androgen-insensitive PC-3 cells (data not shown).

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and harvesting efficiency, cells were cotransfected with pRSVL, a

Fig. 6. RNase protection assay using 32P-labeled antisense PSA and β2-MG RNA probes to measure the induction of PSA mRNA expression in LNCaP 104-S, 104-I, 104-R, and PC-3 cells by 0.1 nM R1881. Total RNA was isolated from LNCaP 104-S cells (passage 15), 104-I cells (passage 60), 104-R cells (passage 95), and PC-3 cells grown in DMEM supplemented with 10% CS-FBS in the absence or presence of 0.1 nM R1881. The protected PSA probe was normalized to the amount of protected β2-MG probe.

<table>
<thead>
<tr>
<th>Cell</th>
<th>% conversion</th>
<th>Fold induction by R1881</th>
<th>Relative luciferase activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>104-S</td>
<td>0.18 (0.03)*</td>
<td>6.79 (1.99)*</td>
<td>37.7 (76 (11.8)*</td>
</tr>
<tr>
<td>104-I</td>
<td>0.12 (0.02)</td>
<td>1.48 (0.29)</td>
<td>12.3 (1.02)</td>
</tr>
<tr>
<td>104-R</td>
<td>0.13 (0.03)</td>
<td>35.3 (2.79)</td>
<td>272 (14.54)</td>
</tr>
</tbody>
</table>

* Numbers in parentheses, SE; n = 5.

Table 1 Induction of CAT activity in LNCaP 104 cells

CAT activity, expressed as percentage conversion of [14C]chloramphenicol to acetylated forms, in LNCaP 104 cells at different passage numbers (indicated by subscript) was measured after cotransfection of MMTV-CAT and pRSVL expression vectors.

10 nM R1881 were used. AR was not detectable in PC-3 cells by immunoblot analysis.

AR Activity in LNCaP 104 Cell Lines. AR transcriptional activity in LNCaP 104 cells was assessed by two methods: (a) induction by androgen of PSA mRNA level as measured by RNase protection assay; and (b) induction by androgen of CAT activity in cells transiently transfected with MMTV-CAT, an expression vector carrying the CAT gene linked with the androgen response element-containing MMTV-LTR (41, 42). The 5' regulatory region of the PSA gene, like the MMTV-LTR, has been shown to contain a functional androgen response element (43). PSA mRNA levels were responsive to R1881 over a range of concentration in LNCaP 104-S and 104-R cells (Fig. 5). However, the induction of PSA mRNA level by R1881 was up to 21-fold higher (with 0.1 nM R1881) in 104-R cells than in 104-S cells. The level of PSA mRNA induction by androgen in 104-S cells is comparable to the induction by androgen of PSA mRNA in LNCaP cells reported by others (37, 44, 45). Induction of PSA mRNA level by 0.1 nM R1881 in LNCaP 104-I cells was intermediate with respect to the induction observed in 104-S and 104-R cells (Fig. 6). The basal level of PSA mRNA expression was slightly higher in untreated 104-I and 104-R cells than in untreated 104-S cells, possibly reflecting the sensitivity of AR in the later stage 104 cells to residual androgen in the medium. AR-mediated induction of PSA mRNA level, however, is clearly androgen-dependent in 104-I and 104-R cells. Expression of PSA mRNA was not observed in PC-3 cells.

An increase in AR activity in LNCaP 104-R cells but not in 104-I cells was observed in LNCaP 104 cells transiently transfected with the MMTV-CAT expression vector (Table 1). To normalize within a cell type for plate-to-plate variation in transfection efficiency, cell number, and harvesting efficiency, cells were cotransfected with pRSVL, a vector in which expression of the luciferase gene is driven by the RSV LTR (40). R1881 induced CAT activity nearly 38-fold in 104-S cells, 12-fold in 104-I cells, and 272-fold in 104-R cells. It is unclear why 104-I cells exhibited the lowest induction of CAT activity, in light of the results with PSA expression. It is possible that the apparent low induction by androgen in 104-I cells reflects differences in the activity of other factors involved in the basal and/or induced transcriptional activity of the MMTV LTR. LNCaP 104-I cells also exhibited the lowest level of luciferase activity (Table 1).

Effect of Androgen on c-myc Expression in LNCaP 104 Cell Lines. Androgen regulation of c-myc expression in LNCaP 104 cells was examined to determine whether expression of this critical growth-related gene changed concurrently with the increase in AR expression and transcriptional activity and with the change in the proliferative response to androgen. Based on RNase protection analysis of c-myc mRNA levels, 0.1 nM R1881 induced the c-myc mRNA level over 2-fold in 104-S cells but reduced the c-myc mRNA level to less than 20% of the control value in 104-R cells (Fig. 7). Treatment of 104-R cells with 0.01 nM R1881 caused a 25% increase over the basal c-myc mRNA level, which was nearly 2-fold higher than the basal level of c-myc expression in 104-S cells. Concentrations of R1881 higher than 0.1 nM reduced c-myc mRNA levels in 104-S cells, consistent with the diminished effect of high R1881 concentration on 104-S cell proliferation. Expression of c-myc in intermediate stage 104-I cells was
Total RNA was isolated from LNCaP 104-S cells (passage 14) and 104-R cells (passage 84) grown in the presence of R1881 at the indicated concentrations. The amount of probes to measure the induction or repression of c-myc expression in LNCaP 104-S and 104-R cells by 0.1 nM R1881 (in 10% CS2-FBS) and 20 nM R1881 (in 10% untreated FBS). Total RNA was isolated from LNCaP 104-S cells (passage 14) and 104-R cells (passage 84) grown in the presence of R1881 at the indicated concentrations. The amount of protected c-myc probe was normalized to the amount of protected β2-MG probe.

Effect of Retroviral c-myc Expression on Androgen Control of LNCaP 104 Cell Proliferation. To determine the effect of nonregulated overexpression of c-myc on androgen control of LNCaP 104 cell proliferation, 104-S, 104-I, and 104-R cells were infected with retrovirus carrying the c-myc gene linked with the enhancer/promoter of the Moloney murine sarcoma virus long terminal repeat. Cell proliferation of infected and uninfected cells in the presence or absence of 20 nM R1881 in 10% untreated FBS was compared (Fig. 9). In uninfected LNCaP 104-S, 104-I, and 104-R cells, cell numbers were reduced to about 50% of the control values by R1881. In cells overexpressing c-myc, R1881 inhibition of proliferation was not observed. The protection from growth inhibition was c-myc dependent since proliferation of LNCaP 104-S cells infected with retrovirus not expressing c-myc was reduced to a similar level by 20 nM R1881 as in uninfected 104-S cells. In comparison with 104-S cells, cells at the later stages of passage (104-I and 104-R), uninfected or retrovirally infected, exhibited a progressive decline in proliferation rate when grown in medium supplemented with 10% untreated FBS. In uninfected cells, this decline may have been due to the fact that the cells at the later passages were more sensitive than 104-S cells to endogenous androgens present in untreated FBS. However, because this progressive decline in proliferation is also observed in cells overexpressing c-myc, the phenomenon may not be mediated through c-myc.

Changes in Morphology of LNCaP 104 Cells. LNCaP 104 cells underwent a change in morphology in the transition from 104-S to 104-I and 104-R stages. LNCaP 104-I and 104-R appeared progressively more rounded and less spindle-shaped than 104-S cells, which resembled the morphology of the parental LNCaP population. In addition, 104-I and especially 104-R cells tended to grow as rounded clusters or islands of cells instead of a uniform monolayer. In this respect, they are similar to the LNCaP FGC clone 22 cells, which were described as an androgen-independent subline by van Steenbrugge et al. (6). However, LNCaP 104-I and 104-R cell clusters do not exhibit the “flattened character” of clone 22 cells.

DISCUSSION

It is well recognized that proliferation of LNCaP cells in steroid-depleted medium is induced at an optimal androgen concentration, above which diminished proliferation is observed (5, 12, 47). Proliferation in untreated FBS-supplemented medium is repressed by moderate to high concentrations of androgen (16), particularly when metabolically stable androgens such as R1881 and DMNT are used. The use of metabolically stable R1881 in this study made it possible to analyze androgen concentration-dependent changes in the biphasic response (6, 47, 48) over the period of androgen deprivation. The fact that the concentration of R1881 required for induction and repression of the proliferation of LNCaP 104 cells gradually decreased as the cells were passaged in steroid-depleted medium suggests that these cells gradually increased their sensitivity to lower concentrations of androgen (Figs. 1 and 2). It is important to note that although optimal induction of proliferation of 104-R cells by R1881 was less than that of 104-S cells (2-fold compared with 10- to 13-fold), optimal induction was observed at a 10-fold lower concentration of R1881. Additionally, the basal proliferation rate of untreated 104-R cells was much higher than that of 104-S cells. The levels of AR mRNA and protein were significantly higher in 104-R cells than in 104-S cells. LNCaP 104-R cells, therefore, can be considered to have enhanced proliferative sensitivity to androgen. Enhanced androgen sensitivity is supported by the progressively increased expression of AR responsive genes, APA, and the MMTV-CAT reporter gene in 104-R cells compared with that in 104-S and 104-I cells. Enhanced sensitivity to R1881 may be the additive result of increased expression of the AR mRNA and protein (Figs. 3 and 4) and increased transcriptional activity of AR in 104-R cells. The approximately 5-fold increase in the AR protein level of 104-R cells over 104-S cells in the presence of R1881 probably cannot account for the 21-fold higher induction of
PSA mRNA (Fig. 5) observed in 104-R cells. Therefore, other possibilities should also be considered. For example, these cells could have changes in AR phosphorylation (49–51), in the activity of transcription factors or AR-associated factors (52–54), and in androgen binding properties which could alter intranuclear localization and the half-life of AR (51, 55).

The increase in AR expression and activity may be viewed as an adaptive response of LNCaP 104 cells to exceedingly low concentrations of available androgen (15). This stands in contrast to the loss of androgen sensitivity in Shionogi 115 mouse mammary tumor cells after androgen withdrawal (4, 56). The increased expression of AR mRNA in 104-R cells after long-term androgen deprivation is consistent with the repressive effect of androgen on AR mRNA levels in LNCaP cells observed after short-term androgen treatment (Fig. 3; Refs. 7, 9, and 45). This adaptive response of LNCaP cells to low androgen concentration offers no information about how some prostate tumor cells and established cell lines such as PC-3 and DU145 have lost AR expression. It is possible that loss of AR expression occurs after alterations in the expression of specific genes involved in control of cell proliferation take place which make AR expression nonessential for cell growth and survival. For example, PC-3 cells have been shown to lack expression of p53 (57), and DU145 cells express an inactive form of the retinoblastoma gene product (57, 58). Recently, Yuan et al. (59) reported that the proliferation of PC-3 cells stably transfected with an AR expression vector is inhibited by androgen. This inhibition may be analogous to the inhibition by androgen of LNCaP 104-R cell proliferation described in this paper.

The induction and repression of cell proliferation by androgen in LNCaP 104 cells appear to be mediated chiefly, although perhaps not exclusively, through a pathway involving the regulation of c-myc expression. Induction and repression of c-myc expression in LNCaP 104-S and 104-R cells by 0.1 nM R1881 correlated directly with the induction and repression of cell proliferation by R1881 at the same concentration, with the exception that no induction of c-myc mRNA level was observed in 104-R cells treated with 0.01 nM R1881. Repression of cell proliferation by 20 nM R1881 was effectively overcome by exogenous retroviral overexpression of c-myc. The mechanism by which androgen receptor regulates c-myc mRNA level in LNCaP cells is not understood. However, Wolf et al. (17) determined by nuclear run-on analysis that the synthetic androgen DMNT repressed c-myc expression in LNCaP cells at the level of transcription initiation. A 3-h lag between the time of DMNT addition and decline in c-myc transcription suggests that the repression mechanism probably involves multiple steps between hormone binding and c-myc transcription. These investigators described a potential glucocorticoid/androgen response element within the first intron of the c-myc gene, but whether AR binds to this site or acts less directly has not been shown. Androgens have been reported to induce the level of EGFR receptor approximately 2-fold in LNCaP cells grown in medium supplemented with charcoal-pretreated FBS (47, 60). LNCaP cells produce EGFR and EGFR-like peptides, but the production appears to be insensitive to androgen treatment (61). Eaton et al. (62) reported a strong correlation of EGFR receptor level with c-myc expression in human prostate carcinoma tissue samples. Basic fibroblast growth factor and fibroblast-derived growth factors have also been reported to promote LNCaP cell proliferation in vitro and in vivo (63, 64). Whether positive modulation of c-myc expression by androgen occurs through pathways involving peptide growth factors and their receptors remains to be shown. Given the complexity of the regulation of the c-myc gene expression, which encompasses control of transcription initiation from multiple promoters and transcription elongation, as well as posttranscriptional control of mRNA stability (20, 21), it is possible that androgen affects c-myc mRNA levels in LNCaP cells through multiple pathways.

The mechanism by which the synthetic androgen R1881 can elicit opposite proliferative responses at different concentrations is not presently understood. However, the idea that the LNCaP cell line is a rare example of a type of prostate tumor, the growth of which is only inhibited by androgen (16), is not consistent with our observation that during the adaptation to low androgen concentration, LNCaP 104-S and 104-I cells progress to 104-R cells characterized by increased AR expression and activity and enhanced sensitivity to androgen. If androgen exerted only negative selective sensitivity to cells deprived of androgen probably would not increase their sensitivity to it over time.

If the type of adaptation in AR expression and activity exhibited by LNCaP 104 cells is a general phenomenon, the clinical implications are significant. Some prostatic tumor cells under the selective pressure of antiandrogen and/or or androgen ablation therapy may be able to adapt to lower androgen concentration (such as the level supplied by adrenals in castrated patients) by increasing the transcriptional activity or steroid affinity of AR and thereby overcome the therapy. Additionally, adaptation to lower androgen availability may allow cancer cells to survive and proliferate during therapy until other events occur which enable cells to bypass the androgen requirement altogether. Proliferation of prostate cancer cells which are hypersensitive to very low concentrations of androgen may in fact be repressed by moderate concentrations of androgen. The molecular mechanisms involved in the transition from 104-S to 104-R stages and the effects of changes in AR expression and activity on regulation of c-myc and other critical growth-related genes in LNCaP cells are currently under investigation.

ACKNOWLEDGMENTS

We thank Ching Song and Richard Hiipakka for assistance in the preparation and purification of the AN-21 antibody.

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Increased Androgen Receptor Activity and Altered c-myc Expression in Prostate Cancer Cells after Long-Term Androgen Deprivation


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