Regulation of Androgen-dependent Prostatic Cancer Cell Growth: Androgen Regulation of CDK2, CDK4, and CKI p16 Genes

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ABSTRACT

Growth of prostatic epithelial cells is androgen-dependent; however, the mechanism of androgen action on cell growth is not well defined. We investigated whether androgen-dependent prostatic epithelial cell growth is mediated by androgen regulation of expression of genes controlling cell cycle progression. For this purpose, we used an androgen-dependent prostatic cancer cell line, LNCaP-FGC, as an in vitro model. We found that expression of CDK2 and CDK4 genes were up-regulated within hours of androgen treatment as detected in Northern and Western blot analyses. Kinase assay also confirmed that there was increased CDK2 kinase activity upon androgen stimulation. Moreover, androgen down-regulated expression of the cyclin-dependent kinase inhibitor p16 (MTSI, CDKN2) gene. The overall effects of these androgen actions result in an increased cyclin-dependent kinase activity and stimulation of the cell to enter S phase of the cell cycle, thereby enhancing cell proliferation. In contrast, an androgen-independent PC-3 cell line lost its response to androgen stimulation, and higher basal levels of CDK2, CDK4, and p16 genes were constitutively expressed in PC-3 cells. Collectively, these data suggest a possible signaling pathway of androgen in stimulating cell growth. These results also imply that in androgen-dependent prostate cancer, increased androgen receptor (AR) activity, resulting from AR gain-of-function mutations, AR gene amplification, or AR gene overexpression, malignantly stimulates proliferation of prostatic epithelial cells and constitutes one possible mechanism of androgen-dependent tumorigenesis.

INTRODUCTION

Prostate carcinoma is the second leading cause of cancer death in men (1). Etiology, progression, and metastasis of prostate cancer are not well understood (2, 3). At the early stage of prostate cancer, growth of prostatic epithelial cells is androgen-dependent. Castration has been a very effective method to treat prostate cancer patients (4). However, it causes only a temporary regression of prostate cancer (3, 5), and some tumor cells become androgen-independent (3, 5). Genetic changes are among the factors contributing to the development and progression of prostate cancer (2, 3). Mutations in transcriptional regulators such as the AR as well as tumor suppressor genes are possible signaling pathways of androgen in stimulating cell growth. These results also imply that abnormally activated AR activity may malignantly stimulate cell proliferation, suggesting one possible mechanism of androgen-dependent tumorigenesis.

MATERIALS AND METHODS

Cell Culture. Human metastatic prostate adenocarcinoma cell line LNCaP-FGC (American Type Culture Collection) was maintained in RPMI 1640 (Life Technologies, Inc.) supplemented with either 10% FBS or 10% charcoal/dextran-treated (stripped) FBS (Hyclone Laboratories) at 37°C in 5% CO₂. Human prostate adenocarcinoma cell line PC-3 (American Type Culture Collection) was cultured in DMEM:F12 (Life Technologies, Inc.) supplemented with either 10% FBS or stripped FBS.

Reagent. AR agonist R1881 was purchased from DuPont Biotechnology Systems. MTt assay was from Sigma. Human CDK4, p15, and p16 cDNA probes were gifts from Dr. David Beach (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY). Human p27, CDK2, and CDK6 CDNA probes were kindly provided by Dr. Wade Harper (Department of Biochemistry, Baylor College of Medicine).

MTT Assay. Effects of androgen on tumor cell growth were assessed by MTT assay as described previously (19). Briefly, LNCaP-FGC and PC-3 cells, which had been cultured in medium supplemented with stripped FBS for 1 week, were harvested by exposure to 0.25% trypsin and 0.02% EDTA (w/v) and seeded into a 96-well microculture plate at a density of 10,000 cells/well in RPMI-10% stripped FBS. After incubation in 5% CO₂ at 37°C overnight, the cells were incubated in the same medium containing AR agonist R1881 (10⁻⁹ M) for 2, 4, and 6 days. At the end of the incubation, 20 μl of MTT (2.5 mg/ml) were added to each well, and the cells were incubated for 2 h at 37°C to allow complete reaction between the dye and the enzyme mitochondrial dehydrogenase in the viable cells. After removal of the residual dye and medium, 100 μl of DMSO were added to each well, and the absorbance at 540 nm was measured with a MRX microplate reader (Dynatech Laboratories).

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2 To whom requests for reprints should be addressed.
3 The abbreviations used are: AR, androgen receptor; CDK, cyclin-dependent kinase; CKI, CDK inhibitor; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; FBS, fetal bovine serum; PBST, PBS containing 0.1% Tween 20.
cells were cultured at the same condition for 4 days. \( ^{3}H \) Tymidine (0.5 mCi/well, 6.7 Ci/mmole; ICN) was added into each well 16 h before the end of the incubation, and the cells were harvested onto a glass filter using a cell harvester. Incorporation of \( ^{3}H \) Tymidine into cellular DNA was measured by liquid scintillation counting.

**Northern Blot Analysis.** Total cellular RNA from control and R1881-treated samples was isolated using Ultraspec RNA isolation reagent (Biotex Laboratories, Inc.). Total RNA (20 \( \mu \)g/sample) was fractionated on a 1% agarose gel and transferred onto a nylon filter (Hybond-N; Amersham). Northern hybridization was performed using Quikhyb hybridization solution according to the manufacturer’s recommendations (Stratagene).

**Western Blot Analysis.** Aliquots of samples with same amounts of protein, determined using the Bradford assay (Bio-Rad), were mixed with loading buffer [final concentrations, 62.5 mM Tris-HCl (pH 6.8), 2.3% SDS, 100 mM DTT, and 0.005% bromphenol blue], boiled, fractionated in a 15% SDS-PAGE, and transferred onto a 0.45-\( \mu \)m nitrocellulose membrane (Bio-Rad). The blots were blocked with 2% fat-free milk in PBS and probed with anti-CDK2 and anti-CDK4 antibodies (0.05 \( \mu \)g/ml IgG; Santa Cruz Biotechnology, Inc.) in PBST and 1% fat-free milk. The membranes were then washed once in PBST and incubated with horseradish peroxidase-conjugated Fab(\( \alpha \))\(_2\) goat antirabbit secondary antibody (Bio-Rad) in PBST containing 1% fat-free milk. After washing four times in PBST, the membranes were visualized using the enhanced chemiluminescence Western blotting detection system (Amersham). Quantitation of each band was performed using IPLab Gel Eval computer program (Signal Analytics Corp.).

**Immune Complex Protein Kinase Assay.** Immunoprecipitation followed by CDK2 kinase assay was performed according to the manufacturer’s recommendations (Santa Cruz Biotechnology, Inc.). Briefly, 1 mg of LNCaP-FGC cell lysate was incubated with 0.5 \( \mu \)g of anti-CDK2 antibody (Santa Cruz Biotechnology, Inc.) for 1 h in 1 ml of radioimmunoprecipitation buffer (PBS, 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, 0.1 mg/ml phenylmethyl-sulfonyl fluoride, 30 \( \mu \)l/ml aprotinin, and 1 mm sodium orthovanadate). Subsequently, 30 \( \mu \)l of protein A-Sepharose 4B conjugate (Zymed Laboratories Inc.) were added, and the mix was incubated at 4\( ^\circ \)C on a rocker platform for 1–24 h. After washing the immune complexes four times in radioimmunoprecipitation buffer, the beads were resuspended in 23 \( \mu \)l of kinase buffer [50 mM Tris-Cl (pH 7.5), 10 mM MgCl\(_{2}\), and 1 mM DTT]. Two \( \mu \)l of histone H1 (Boehringer Mannheim), 1 \( \mu \)M ATP, and 20 \( \mu \)Ci of \(^{32}\)P]ATP (7000 Ci/mmol) were added to a final volume of 25 \( \mu \)l. The reaction was incubated at 30\( ^\circ \)C for 30 min and stopped by the addition of SDS sample buffer. After boiling, samples were electrophoresed through a 12% SDS-polyacrylamide gel. The gel was then exposed to X-ray film.

**RESULTS**

Androgen-dependent Growth of Prostatic Epithelial Cells. We studied androgen-dependent cell growth using LNCaP-FGC cells (5). LNCaP-FGC cells express a high level of the gain-of-function mutated form of AR, in which AR is not only activated by androgen but also by progesterone, estradiol, and antiandrogens. In contrast, PC-3 cells are an androgen-independent prostatic adenocarcinoma cell line that expresses a very low or undetectable level of AR and undetectable responses to androgen (20). In the first set of experiments, we verified the androgen-dependent growth of these two cell lines. After culturing the cells for 7 days in medium containing 10\% stripped FBS, cell growth in response to androgen stimulation was determined by MTT assay. In the absence of AR agonist R1881, LNCaP-FGC cells did not grow (Fig. 1A). Growth of LNCaP-FGC cells was stimulated by AR agonist R1881 (10\(^{-9}\) M) in a time-dependent fashion, and LNCaP-FGC cell proliferation was dramatically enhanced approximately 3-fold. In contrast, PC-3 cells grew equally well in the absence or presence of androgens (Fig. 1B). These results demonstrated that the LNCaP cell line is a suitable model to study the molecular mechanism of androgen action on cell growth.

A \(^{3}H \) Tymidine incorporation experiment was then used to determine whether androgen can stimulate LNCaP-FGC cells to enter the S phase of the cell cycle. We found that a concentration of R1881 as low as 10\(^{-11}\) M can dramatically stimulate DNA synthesis in LNCaP-FGC cells (Fig. 2). The dose-response curve reached a plateau with further increased concentrations of R1881. This result suggested that androgens can stimulate prostatic epithelial carcinoma cells to enter the S phase and begin DNA synthesis.
Regulation of CDK2 and CDK4 Genes by Androgen. To understand the underlying mechanisms of androgen regulation of cell growth, we investigated whether androgens regulate genes controlling cell cycle progression using Northern blot analysis under the experimental conditions outlined previously. We found that basal levels of CDK2 mRNA in LNCaP-FGC cells were increased 8 h after R1881 stimulation and continued to increase thereafter to 48 h (Fig. 3). This effect of androgen was also observed for CDK4 mRNA in LNCaP-FGC cells. In contrast, PC-3 cells constitutively expressed higher basal levels of CDK2 and CDK4 mRNAs compared with those in LNCaP-FGC cells. Steady-state levels of CDK2 and CDK4 mRNAs were not altered by R1881 treatment in PC-3 cells (Fig. 3). Neither LNCaP-FGC cells nor PC-3 cells expressed detectable levels of CDK6 mRNA in the presence and absence of R1881 (data not shown). As a control, expression of the tumor suppressor gene product p53 was examined. We found that LNCaP-FGC cells expressed high levels of p53 mRNA that were not regulated by R1881. Interestingly, PC-3 cells did not express p53 with or without R1881 (Fig. 3). Similarly, expression of the E2F and Rb genes was not altered in response to androgen stimulation (data not shown).

Next we determined CDK2 and CDK4 protein levels using Western blot analysis. Consistent with Northern blot data, an approximately 2-fold increase in the protein levels of the CDK2 and CDK4 genes was detected in LNCaP-FGC cells incubated for 8 h with R1881 (Fig. 4) and increased to 24 h.

These increased expressions of CDK2 and CDK4 genes should confer enhanced protein kinase activities of CDK2 and CDK4. CDK2 was chosen to do the immune complex kinase assay using histone H1 as substrates. Consistent with Northern blot and Western blot analyses, by 8 h of R1881 stimulation, CDK2 kinase activities were also dramatically increased, as demonstrated by enhanced phosphorylation levels of histone H1, and continued to 24 h (Fig. 5).

Regulation of CKI pi6 Gene by Androgen. Because CKIs are key regulators for the activities of cell cycle genes CDK2, CDK4, and CDK6, we determined whether R1881 regulated the expression of this class of genes. Northern blot analyses indicated that R1881 down-regulated the expression of the CKI pi6 (also called MTS1, CDKN2, and INK4a) gene in LNCaP-FGC cells (Fig. 6). The two different mRNAs encode β and α transcripts generated from separate pi6 gene promoters (21). A significant reduction of pi6 mRNA was observed in cells treated with R1881 for 24 and 48 h (Fig. 6A). The protein level of the pi6 gene was also decreased in response to R1881 stimulation (Fig. 6B). PC-3 cells constitutively expressed higher basal levels of pi6 mRNA (Fig. 6). However, its expression was not altered by R1881 treatment. We also examined the expression of pi5 (also called INK4b) and p27 (also called KIP1) mRNAs in these two cell lines. In contrast to that observed for pi6, we found that expressions of these two genes were not affected by R1881 in either LNCaP-FGC or PC-3 cells.
ANDROGEN REGULATION OF CDK2, CDK4, AND p16 GENES

A. Time (hr) of R1881 (10^{-8}M) treatment

Histone H1

B. Time (hr) of R1881 (10^{-8}M) treatment

Fig. 5. Determination of altered CDK2 kinase activity regulated by androgen. A, after LNCaP-FGC cells were cultured in medium containing 10% stripped FBS for 1 week, androgen agonist R1881 (10^{-8}M) was added for 0, 2, 8, and 24 hr. Subsequently, cell lysates were prepared, and 1 mg of cellular protein from each sample was incubated with 0.5 μg of anti-CDK2 antibodies. The immune complexes were pulled down by protein A-Sepharose 4B, and CDK2 kinase activities were determined using histone H1 as substrates. The reactions were fractionated on a 12% SDS-PAGE followed by exposure to X-ray film. B, quantitative analysis of CDK2 kinase activity.

DISCUSSION

In the present study, we investigated whether androgens could regulate genes controlling cell cycle progression. The LNCaP-FGC cell line was used as an in vitro model by taking advantage of its androgen-dependent growth properties. Androgen-dependent growth of these tumor cells mimics the characteristics of prostatic epithelial cells' growth in vivo (3). Androgen-independent growth of PC-3 cells is indicative of advanced prostate cancer, and these cells are used as a control in our studies (20). It has been shown that LNCaP-FGC cells express a gain-of-function mutation of AR at a very high level (6, 7, 22), whereas PC-3 cells express a low or undetectable level of AR. Our results indicate that androgen up-regulates the expression of the CDK2 and CDK4 genes and down-regulates the expression of the CKI p16 gene in LNCaP-FGC cells. The consequence of these altered gene expressions in LNCaP-FGC cells is to stimulate cells to enter the S phase of the cell cycle and thus increase cell proliferation. The molecular mechanisms of these altered gene expressions on androgen stimulation remain to be determined.

CDKs are cell cycle enzymes tightly regulated by association with cyclins and protein phosphorylation (23). Sequential activation of the cyclin-CDK complexes is thought to be responsible for orderly transitions through the cell cycle. Abnormal activation of CDK activity, through a variety of mechanisms, may underlie part of the uncontrolled growth that characterizes cancer (18). Both CDK4 and CDK6 genes are overexpressed in tumor cell lines derived from breast carcinomas (MCF-7, HBL-100, and BT-549) and leiomyosarcomas (SKUT-1-B; Ref. 24). Furthermore, the CDK4 gene is amplified in tumors such as the osteosarcoma cell line OsAVL and the rhabdomyosarcoma cell line SJRH30 (25). We observed that PC-3 cells contain high levels of CDK2 and CDK4 mRNAs that are independent of androgen stimulation and may contribute to the androgen-independent growth.

Checkpoint regulation of the cell cycle involves CKIs, which potentially act as tumor suppressor genes. Two classes of genes whose products can inhibit CDK activities have recently been identified. The first class consists of p21 (also called WAP1, CIP1, SDII, and CAP20; Refs. 26–28), p27 (also called KIP1; Refs. 29 and 30), and p57 (also called KIP2; Refs. 31 and 32), which possess considerable sequence similarity and can inhibit a variety of CDK subtypes. This class of genes is mainly involved in development (33, 34), because mutations in this class of genes have not been identified in tumors. The second class of CKIs is represented by p16 (35), which encodes a protein with ankyrin-like repeats that can inhibit CDK4 and CDK6. This class of genes also includes p15 (36) and p18 (37). Recently, it has been shown that many tumors such as malignant melanomas, gliomas, lung cancers, and leukemia contain hemizygous or homozygous deletions of chromosome 9p21, in which both p16 and p15 genes reside (38–41). Mutations of the p16 and p15 genes are also found in these...
types of tumors, suggesting a tumor suppressor function for this class of genes. We found that androgen down-regulates the expression of the p16 gene at both the mRNA level and the protein level, implying that the hormone can release the inhibitory effect of the gene for the p16 gene at both the mRNA level and the protein level, implying that the hormone can release the inhibitory effect of the gene for p16 androgen action and cell cycle regulation, implying a molecular mechanism for the down-regulation of CDKs in prostatic epithelial cells. Additional studies are needed to elucidate the molecular mechanisms of these androgen regulations.

The potential significance of this study underlies the connection of androgen action and cell cycle regulation, implying a molecular mechanism of androgen action on cell growth. As illustrated in Fig. 7, we propose that any mechanism that can abnormally increase AR activity may contribute to the enhanced growth of prostatic epithelial cells and potentiate tumorigenesis. The gain-of-function mutations of the AR gene, such as those in codons 715 (Val—Met), 877 (Thr—Ala), and 1042 (Arg—Cys), result in enhanced AR activity. Such mutations can also result in enhanced AR activity.

In summary, this study connects androgen action and cell cycle control, which may regulate the proliferation and differentiation of prostatic epithelial cells. In prostate cancer, abnormally increased AR activities by ligand-dependent pathways such as AR gain-of-function mutation, AR overexpression, and AR gene amplification as well as the ligand-independent pathway may increase the rate of cell cycle progression and contribute to the enhanced growth of prostatic epithelial cells.

REFERENCES

ANDROGEN REGULATION OF CDK2, CDK4, AND p16 GENES


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