Prostate Cancer Cell Cycle Regulators: Response to Androgen Withdrawal and Development of Androgen Independence

David B. Agus, Carlos Cordon-Cardo, William Fox, Marija Drobnjak, Andrew Koff, David W. Golde, Howard I. Scher

Background: Androgen withdrawal is a standard therapy for prostate cancer that results in a decrease in tumor volume and a decline in serum prostate-specific antigen in the majority of patients. To understand the factors associated with regression of prostate cancers after androgen withdrawal, we studied cell cycle regulator changes in the CWR22 human prostate cancer xenograft model. Methods: Established tumors in nude athymic BALB/c mice were sampled at various times after androgen withdrawal and after the development of androgen independence. Changes in the expression of cell cycle regulators were categorized into early and mid-to-late events. Results and Conclusions: Early events included a decrease in androgen receptor expression, followed by a short-term increase in expression of the p53 and p21/WAF1 proteins and a marked decrease in the Ki67 proliferative index. Mid-to-late events included progressive and sustained increases in p27 and p16 protein expression, a decrease in retinoblastoma protein expression, and an increase in the transcription factor E2F1. Changes in apoptosis (programmed cell death) were not observed at any time after androgen withdrawal. These data suggest that androgen withdrawal results in a cell stress response, in which increased p53 protein produces a cell cycle arrest, without activation of p53-mediated apoptosis. The proliferative index is further decreased through the action of the cyclin-dependent kinase inhibitors p27 and p16. Androgen-independent sublines emerged 80–400 days after androgen withdrawal, and these sublines had variable growth phenotypes but were associated with mdm2 protein overexpression and increased expression of cyclin D1. These results indicate that tumor regression in this human prostate cancer model is due to cell cycle arrest rather than to apoptosis and that the emergence of androgen independence is associated with a release from cell cycle arrest. [J Natl Cancer Inst 1999;91:1869–76]

Prostate cancers have a low proliferative index and depend on androgens for growth. After castration, these tumors regress but later progress to an androgen-independent phenotype that is ultimately lethal for the patient. Knowledge of the mechanisms associated with the inability of androgen withdrawal strategies to result in the complete regression of a prostate cancer and the mechanisms that contribute to androgen independence would have valuable therapeutic implications. Previously, we showed that androgen withdrawal in mice bearing the CWR22 human prostate cancer xenograft (1–3) was associated with a decrease in the proliferative index (4). Cellular changes consistent with apoptosis (i.e., programmed cell death) were rare at the times evaluated. Thus, this study was undertaken to determine dynamic changes in the expression of critical cell cycle regulators, in the p53 pathway, and in the retinoblastoma protein pathway, associated with the response to androgen withdrawal and with the emergence of the androgen-independent phenotype.

Reproducible changes in expression of cell cycle regulators were found that could be categorized into early and mid-to-late events. Early events were consistent with a cell stress response in which p53 produces a transient cell cycle arrest without activation of p53-dependent cell death programs. Mid-to-late events included an increase in the expression of the cyclin-dependent kinase inhibitors p27 and p16, which are associated with a further decline and the maintenance of a low proliferative index. Apoptotic changes were not observed after androgen withdrawal. Characterization of the androgen-independent phenotype revealed the overexpression of mdm2, affecting p53 stability, as well as the increased cyclin D1 expression, affecting pRB phosphorylation. These results challenge a commonly held view that the regression of prostate cancers after androgen withdrawal is mediated exclusively by apoptotic mechanisms. Furthermore, the results suggest that therapeutic strategies directed at the cell cycle-arrested prostate cancer cells, after androgen withdrawal, may be clinically important.

Materials and Methods

Animal Studies

Four- to 6-week-old nude athymic BALB/c male mice were obtained from the National Cancer Institute-Frederick Cancer Research and Development Center, Frederick, MD, and maintained in pressurized, ventilated caging. Institutional guidelines for the proper and humane use of animals in research were followed. The CWR22 tumor line was propagated in the animals by the injection of a mixture containing recombinant basement membrane (Matrigel; Collaborative Research, Inc., Bedford, MA) and minced tumor tissue from an established tumor into the subcutaneous tissue of the flanks of athymic nude mice (1,2). For the maintenance of serum androgen levels, mice were administered 12.5 mg of sustained-release testosterone pellets (Innovative Research of America, Sarasota, FL) subcutaneously. Tumors of approximately 1.5 × 1.0 × 1.0 cm grew 3–4 weeks after inoculation. Androgen withdrawal was accomplished by surgical castration under pentobarbital anesthesia and removal of the testosterone pellets. Tumor size was determined by caliper measurements of height, width, and depth. Prostate-specific antigen (PSA) assays were performed on the serum of the mice obtained by tail bleeding and used a Tandem-R PSA immunoradiometric assay (Hybritech, Inc., San Diego, CA). Androgen-independent sublines of the parent CWR22 were obtained by following tumors for regrowth and increases in serum PSA after androgen withdrawal. The androgen-independent sublines regrew 80–400 days after androgen withdrawal. The sublines were serially passaged in three castrated hosts before characterization as a subline. These were duplicates performed at each sampling point in the experiments.

Histopathology, Monoclonal Antibodies, and Immunohistochemistry

Tissues were fixed in 10% buffered formalin and embedded in paraffin, and sections (5 μm) were stained with hematoxylin–eosin. The following well-characterized antibodies were used at the corresponding final working dilutions: anti-Ki67 mouse monoclonal antibody (MAB) MIB1 (Immunotech, SA, Marseille, France; 1:50 dilution), p53 (MAB clone PB18101; Calbiochem/Oncogene Sciences, Cambridge, MA; 0.2 μg/μL), p21/WAF1 (MAB clone 2G12; PharMingen, San Diego, CA; 0.5 μg/
was graded as follows: 0, undetectable; 1+, minimal staining. The intensity of staining was evaluated more than 500 cells. Data were recorded in a continuous spectrum for 30 minutes (Vector Laboratories, Inc.; 1:25 dilution). Diaminobenzidine was used as the chromogen, and hematoxylin was used as the nuclear counterstain. Most markers were evaluated using 0.01% citric acid (pH 6.0) for 15 minutes, allowed to cool, and incubated with primary antibodies overnight at 4 °C. Biotinylated horse anti-mouse IgG antibodies and biotinylated goat anti-rabbit antibodies were used. The signal was amplified by avidin–biotin peroxidase complexes for 30 minutes (Vector Laboratories, Inc.; Santa Cruz, CA) with a multicycle soft program for assessment of the method of terminal deoxynucleotidyl transferase-mediated uridine triphosphate end labeling (TUNEL) activity in vitro as described by Gavrieli et al. (6), originally described by Gavrieli et al. (7). Briefly, after the exposure of nuclear DNA of tumor extracts were assayed for p27 degradation activity in vitro as described by Nguyen et al. (9). Digoxigenin-labeled probes were used for in situ hybridization, and 1 µg of recombinant plasmid pCR™II (Invitrogen Corp., San Diego, CA), containing the full-length human p27KIP1 gene, was linearized by digestion with BamHI and XbaI to generate antisense and sense transcripts. Each assay was done in duplicate.

**RESULTS**

**Dynamic Cell Cycle Changes After Androgen Withdrawal**

**Early events.** Androgen withdrawal was associated with an abrupt decrease in androgen receptors, detected by nuclear staining, from 60% (1+ ) of tumor cells positive on day 0 (baseline) to 10% by days 3, 7, and 10 (Fig. 1, A and B). The proportion of tumor cells with nuclear staining for p53 increased from a few scattered nuclei in less than 5% of cells on day 0 to approximately 30% (1–2+ ) on day 3 (Fig. 1, C and D; maximal staining). The p53 staining decreased to baseline by day 7. After the increase in p53-positive cells, the number of cells expressing p21/
WAF1 increased from approximately 25% (day 0) to a maximum of approximately 50% of the cells (1–2+) by day 5 (Fig. 1, E and F) and thereafter gradually decreased. Concurrent with the increase in p21/WAF1, a substantial increase in p16INK4A was noted. At days 0–2 after androgen withdrawal, 5%–10% of cells were p16 positive (1–2+); this percentage increased to 30% on day 3 and to 40% on days 5–7 (1–2+). The net effect of these early changes was a decrease in the Ki67 proliferative index, which ranged from 60% to 65% on days 0–3 to 20% on day 5 to approximately 5% on day 7 (Fig. 1, G and H). Levels of cyclin D1 expression also fell from 10% of tumor cells (pretreatment and day 0) to approximately 2% on day 7 after androgen withdrawal.

Expression of mdm2 and bax, other p53-transactivated proteins, did not change during this early period. In fact, expression of mdm2 and bax ranged from 1% to 5% before and after androgen withdrawal. In addition, we did not detect bcl-2 in prostate cancer cells before or after androgen withdrawal (data not shown). The apoptotic rate, as assayed by TUNEL, ranged from 0.2% to 0.8% during the first 7 days (data not shown). Flow cytometry analysis of the CWR22 tumor cells stained with propidium iodide showed a decrease in the percentage of cells in the S-phase fraction from 22% before androgen withdrawal to 5.8% 5 days after androgen withdrawal and less than 3% by 7–10 days (data not shown).

Thus, early cell cycle events after androgen withdrawal are characterized by exit from the cell cycle (summarized in Fig. 2, A), which is associated with a cellular stress response that is initiated by an increase in p53, followed by an increase in p21/WAF1 and a resultant early G1/G0-phase arrest, with the lack of an apoptotic response. Concurrent with these changes after androgen withdrawal, the level of...
PSA fell because PSA transcription is driven by androgen (Fig. 2, B) (4).

Mid-to-late events. Early events were followed by a change in the expression of pRB. Before androgen withdrawal, the intensity of pRB staining was strong (2+), and pRB staining was detected in more than 80% of the cells (Fig. 3, A). On about day 5 after androgen withdrawal, pRB staining was detected in approximately 50% of cells and the intensity had also decreased (1–2+). By day 10, weak-to-moderate immunoreactivity (1+) was observed in 40%–50% of cells (Fig. 3, B). Western blotting of protein extracts obtained revealed that the intense homogeneous pRB staining was associated with the hyperphosphorylated pRB products (data not shown). In parallel with the decrease in pRB expression, there was a slow and progressive increase in E2F1 expression. E2F1-positive staining ranged from weak (1+) in 20% of the tumor cells on day 0 (Fig. 3, C) to moderate (1–2+) in 50% of the tumor cells on day 10 (Fig. 3, D) to strong (2+) in more than 70% of the tumor cells by day 20. Concomitant with the rise in E2F1 staining, we observed a distinct and sustained increase in p27 levels. Expression of p27 was low to undetectable (0–1+) in the tumor cells before androgen withdrawal (Fig. 3, E) but was detected in 5%–7% (1+) of the cells in the 3 days after castration. p27 was detected on day 5 in 40%–50% of the cells, on days 7–10 in 60%–70% (Fig. 3, F), and on days 15, 20, and 25 in 80%–90% (2+). The level of p27 transcript, as assayed by in situ hybridization, did not change by the withdrawal of androgen (data not shown). Degradation of p27 in the tumor was assessed and found to be androgen dependent. In the presence of androgen, there was substantial p27 degradation, but this activity declined after androgen withdrawal and paralleled the percentage of cells expressing p27 (Fig. 3, G). Because p27 degradation is a possible marker for S-phase cells, the amount of p27 degradation should be associated with the proliferation of cells (9). Expression of p16 continued to increase from approximately 40% of tumor cells at day 7 to more than 60% at day 10. This expression was sustained to day 30 (Fig. 2, A). The mid-to-late changes in cell cycle regulators after androgen withdrawal were associated with a low Ki67 proliferative index. On day 7, the Ki67 index was approximately 5%, decreased to 0.8% and 0.2% on days 10 and 15, respectively, and was undetectable at days 20 and 25. Cyclin D1 was undetectable from days 7 through 25 but was present in the androgen-independent xenografts.

Between week 1 and week 3 after androgen withdrawal, mdm2, bax, and bcl-2 levels did not change relative to those before androgen withdrawal. Also, the rate of apoptosis, determined by the TUNEL assay, was low, ranging from 0.7% to 1.3% between day 7 and day 25 (data not shown). Flow cytometry assays of propidium iodide-stained cells revealed a decrease in the S-phase fraction from 4% on day 7 to less than 1% 15 days after androgen withdrawal (data not shown).

After androgen withdrawal, the mid-to-late cell cycle events are, thus, characterized by a progressive and sustained decrease in cyclin D1 expression, followed by changes in pRB expression (summarized in Fig. 2, A). Growth arrest of the prostate cancer cells appears to be maintained by steady levels of p27 and p16. The prostate cancers did not appear to undergo apoptosis after androgen withdrawal but rather underwent a G1/early G1-phase cell cycle arrest.

Cell Cycle Protein Changes Associated With Androgen Independence

Similar to previous observations (2), androgen-independent proliferation was observed after 80–400 days, with a serum PSA rise preceding tumor volume changes (Fig. 2, B). The pattern of expression of cell cycle regulatory proteins in the five androgen-independent tumors, designated CWR22R, CWRSA1, CWRSA3, CWRSA4, and CWRSA6, reflected a highly proliferative tumor, similar to the CWR22 xenografts. Androgen-independent tumors had a Ki67 proliferative index of 60%–70%, a homo-

---

**Fig. 3.** Sections of tumors from a nude mouse with CWR22 human prostate cancer xenograft immediately before and after androgen withdrawal. Panels A and B: Representative areas of CWR22 tumor sections from days 0 and 10, respectively, after androgen withdrawal. Sections are stained with an anti-retinoblastoma protein monoclonal antibody (MAB). Panels C and D: Representative areas of CWR22 tumor sections from days 0 and 10, respectively, after androgen withdrawal. Sections are stained with an E2F1 MAB. Bars = 100 μm. Panel G: p27 degradation is androgen dependent. The amount of substrate p27 for the reaction is shown in the lane labeled “Input.” Numbers above each lane represent the percent of the input p27 remaining after 2 hours.
geneous and intense staining for androgen receptor in 60%–80% of CWR22R, CWRSA1, and CWRSA4 cells or 90% of CWRSA3 and CWRSA6 cells, and, compared with the growth-arrested tumors, an increase in the proportion of pRB-positive cells with strong nuclear staining and a decrease in E2F1 (data summarized in Fig. 4, A). There was a decrease in the proportion of cells staining for p27, from 80%, when proliferation was low (day 25), to approximately 5% in the androgen-independent tumors. Consistent with these findings, there was a decrease in the proportion of cells staining for p16 from 60% to less than 3%.

Two important differences between the androgen-independent sublines and the parental CWR22 xenograft included cyclin D1 and mdm2 overexpression. Cyclin D1 nuclear reactivity was detected in 20%–25% and mdm2 nuclear overexpression in approximately 30% of the androgen-independent cells (Fig. 4, B). We did not observe notable changes in p53 (undetectable to 1% positive), bax (<5% positive), bcl-2 (undetectable), or apoptotic rate, as assessed by TUNEL assay, in the androgen-independent tumor specimens consistent with the parental CWR22.

The level of androgen receptor expression was 1+–2+ in 60%–80% of the tumor cells of most androgen-independent sublines. Two sublines, CWRSA3 and CWRSA6, expressed higher levels of androgen receptor in a greater percentage of tumor cells (≥90%, 2+ staining). The growth characteristics of these sublines were similar to those of the other sublines, except that their growth was repressed, rather than stimulated, in the presence of physiologic concentrations of androgen. The emergence of androgen independence in this model system is associated with a return of tumor cells to an active cell cycle and increased expression of the oncogenic proteins cyclin D1 and mdm2. In addition, the androgen-repressible growth phenotype is associated with relative androgen receptor overexpression.

DISCUSSION

The current dogma is that androgen withdrawal in prostate cancer will result in the apoptotic death of a majority of cancer cells and that the few remaining cells are resistant and will grow back with an androgen-independent phenotype. Characterization of the androgen-independent phenotype has been limited by the lack of biopsy material from these patients. The CWR22 xenograft model recapitulates a subset of human prostate cancers in that, after androgen withdrawal, there is a decline in PSA levels. This decline is followed by regression of the tumor, a rise in PSA levels, and then regrowth as an androgen-independent neoplasm (2). In this study, we categorized changes in cell cycle regulators after androgen withdrawal as early and mid-to-late events. The early cell cycle events were consistent with a cellular stress response associated with an increase in p53, followed by an increase in p21, and a transient early G1/G0-phase arrest, as shown by a decrease in Ki67 expression and a reduction in the number of cells in S phase. The lack of an apoptotic response during this period is consistent with the low-to-undetectable levels of bax and bcl-2 proteins. These data suggest that the growth arrest observed after androgen withdrawal is the result of a failure to activate cell death mechanisms in this xenograft model and that activating p53-independent cell death signals may be an important strategy to effect a complete response to androgen withdrawal.

The initial cell stress response in this system (an increase in p53 and p21 expression and a block in G1-phase cell cycle progression) is similar to that observed after exposure of cells to high-dose UV radiation (11). Ectopic expression of decorin (12) and treatment of several hu-
man cancer cell lines with mimosine and aphidicolin (13) have also been reported to induce increments of p53 and p21 expression, leading to permanent growth arrest. This p21-mediated G1-phase arrest has been shown to be dependent on functional pRB (14).

After androgen withdrawal, tumor regression appears to result from undetectable cell death or, more likely, from changes in cell volume. The increase in p53 staining at day 3, in the absence of apoptosis, suggests a defect in p53-mediated apoptosis. Androgen withdrawal results in the apoptosis of nonmalignant prostate epithelial cells and is associated with a decrease in cell size (15,16). Studies of xenografts and spontaneous rat prostate cancers have been inconclusive. Increased apoptotic indices were observed in PC-82 and LuCaP human prostate cancer xenograft models (17,18), whereas decreased indices were observed in the Dunning R3327/PAP rat model after androgen withdrawal (19,20). Conflicting results have been observed in human prostate cancers after androgen withdrawal, since increased levels of apoptosis have been reported by some groups of investigators (21–23) but not by other groups (24–27). The apparently contradicting results may reflect the small proportion of cells actually undergoing apoptosis at any one time, the inability to sample tumors repeatedly at different times, or the small overall contribution of apoptotic cell death to human tumor regression. An alternative explanation is that the cell cycle checkpoint status of the various tumors is different. The integrity of the checkpoint status can change the response to anticancer therapy from cell cycle arrest to cell death in the xenograft model system (28).

Although changes in early cell cycle events were transitory, the mid-to-late responses were progressive and sustained. We found a decrease in cyclin D1 levels followed by changes in pRB expression, changes similar to those described for butyrate-induced G1-phase arrest (29). Butyrate inhibits the mitogen-dependent transcriptional induction of cyclin D1 and phosphorylation of pRB (30). Withdraw-androgen removes a mitogenic signal for cyclin D1, resulting in pRB hypophosphorylation and G1-phase arrest. Additional factors associated with the maintenance of arrested growth are the observed overexpression of p21, p27, and p16.

The inhibition of cell cycle progression and the antiproliferative activity of rapamycin have been reported to be dependent on p27 (31). More recently, it has been reported that the protein kinase inhibitor staurosporine induces a G1-phase arrest in cells expressing functional pRB (32). In staurosporine-treated cells, the levels of p27 increase, preventing activation of cyclin E/cyclin-dependent kinase-2 and contributing to the G1-phase arrest. In hormone-naïve primary prostate cancer, low-to-undetectable p27 levels were associated with poor clinical outcomes, independent of tumor stage and grade (10,33–36). However, in one study (10), androgen-independent metastatic bone lesions had low levels of p27, consistent with the xenograft findings. Cellular responses to stress, such as hyperthermia, have been shown to induce p16 and to arrest cells in G1/G0 phase in a p53-independent manner (37). Furthermore, the expression of p16 induces decreased transcription of the RB gene (38).

Androgen independence was marked by the expression of the oncogenic proteins cyclin D1 and mdm2. The increased mdm2 expression increases p53 transcriptional transactivation (39) and also inactivates pRB (40). Cyclin D1 is a pivotal molecule in cell cycle regulation. Its overexpression has been shown to participate in the oncogenesis of many tumors (41,42).

Androgen receptor expression decreased substantially after androgen withdrawal. The mechanism of this decrease is not known, but this observation has been made in other systems (43). The androgen receptor in the CWR22 model has a mutation in the ligand-binding domain at position 874 (44). Similar to the wild-type androgen receptor, the receptor is functional in the presence of testosterone and dihydrotestosterone (44). Androgen receptor expression is present again at days 10–16 after androgen withdrawal and to various amounts in androgen-independent sublines. These results are similar to the human condition, where androgen receptor is expressed in the majority of untreated prostate cancers and in most patients whose cancer recurs during androgen withdrawal therapy (45). Other investigators (46,47) have shown that amplification and the increased expression of a wild-type androgen receptor gene may play an important role in androgen-independent prostate cancer. It is of interest that the two androgen-independent tumors with the highest number of cells staining for androgen receptor and the most intense staining showed androgen-repressed growth. Androgen repression of prostate cancer growth has been described previously (48–50). Similar to the current results, androgen-repressible lines expressed higher basal levels of androgen receptor protein and messenger RNA than did the parental line (50). The overexpression of androgen receptor in androgen-independent prostate cancer may identify a subset of prostate cancers in which androgen treatment may be of clinical benefit.

This study demonstrates that reproducible cell cycle protein regulator changes occur with androgen withdrawal in one xenograft model of human prostate cancer. These changes were associated with a cell stress response and the exit of the prostate cancer cells from the active cell cycle with the absence of observed apoptosis. The cell cycle changes associated with the emergence of androgen independence are more heterogeneous but suggest regulatory pathways for this independence from hormonal stimulation. Therapeutic strategies designed to address the cell cycle-arrested prostate cancer cells after androgen withdrawal are being investigated in an effort to eliminate the emergence of androgen independence.

References

6. Fuks Z, Persaud RS, Alfieri A, McLooglin M, Ehlert D, Schwartz H, et al. Basic fibroblast growth factor protects endothelial cells against...
(37) Kokontis JM, Hay N, Liao S. Progression of LNCaP prostate tumor cells during androgen...


NOTES

Supported in part by Public Health Service grants RO1CA30388 (National Cancer Institute) (to D. W. Golde), RO1HL42107 (National Heart, Lung, and Blood Institute) (to D. W. Golde), and DK47650 (National Institute of Diabetes and Digestive and Kidney Diseases) (to C. Cordon-Cardo), National Institutes of Health, Department of Health and Human Services; and the PepsiCo Foundation and CaP CURE (H. I. Scher and D. B. Agus). D. B. Agus is supported by a Physician’s Research Training Award from the American Cancer Society, a CaP CURE Young Investigator Award, and the Eleanor and Paul Stephens Foundation.

Manuscript received January 29, 1999; revised August 4, 1999; accepted August 30, 1999.