Overexpressed Androgen Receptor Linked to p21WAF1 Silencing May Be Responsible for Androgen Independence and Resistance to Apoptosis of a Prostate Cancer Cell Line

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ABSTRACT

An androgen-independent (AI) prostate cancer cell line, derived recently from an LNCaP cell line maintained in androgen-poor conditions, has properties resembling a subgroup of advanced prostate cancers in that it has an overexpressed androgen receptor (AR), undetectable levels of p21WAF1 and prostate-specific antigen, and is resistant to apoptosis. The loss of prostate-specific antigen expression but not the p21WAF1 is attributable to gene silencing by hypermethylation. The high AR and undetectable p21WAF1 of AI cells, and lower AR but highly expressed p21WAF1 of androgen-dependent parental LNCaP cells, suggest a possibility of a functional link between these two proteins. Therefore, we examined the impact the modulation of AR will have on the expression of p21WAF1. Treatment of androgen-dependent cells with an androgen agonist, R1881, increased the AR protein level, whereas it simultaneously reduced the endogenous p21WAF1 protein 8-fold and the activity of a transiently transfected p21-promoter-reporter 10-fold. The down-regulation of p21WAF1 promoter appeared to be ARE mediated, dependent on AR, and not cell-type specific. Furthermore, a reduction of the AR level in AI cells by AR-antisense oligonucleotide increased the p21WAF1 promoter-reporter activity by 4-fold, confirming a functional link between these two proteins. A strong, direct induction of p21WAF1 expression achieved by treatment of AI cells with trichostatin A produced a partial reversion of the AR phenotype evidenced by increased sensitivity of these cells to paclitaxel-induced apoptosis. Moreover, a reduction of AR level by antisense treatment, which also increased p21WAF1 expression, partially restored the androgen dependence of AI cells for growth. The functional link between AR dosage and p21WAF1 expression suggests that therapeutic reduction of AR protein in advanced prostate cancers with elevated AR levels may re-establish their hormone dependence and improve therapeutic response to repeated hormonal ablation and/or induction of apoptosis.

INTRODUCTION

Interaction of the AR with its ligand androgen plays a critical role in growth and development of both normal prostate and prostate cancer (1). Because the observations by Huggins and Hodges (2) and Huggins et al. (3) in the early 1940s on the benefit of androgen ablation for prostate cancer patients, hormonal therapy remains a major approach to treatment. Through block in production of the AR ligand, this treatment interrupts cellular pathways responsible for growth stimulation of tumor cells inducing growth arrest and/or apoptosis (4). However, almost invariably the tumors progress to an AI and apoptosis-resistant state. Maintaining or re-establishing the sensitivity to hormonal therapy and chemotherapy could constitute a major advance in prolonging lives of prostate cancer patients. To that aim we have recently derived an AI prostate cancer cell line from the AD LNCaP cells (5). As shown previously by us (5) and others (6), the AI cells overexpress AR and are more resistant than the parental AD cells to apoptosis-inducing treatments, resembling the vast majority of prostate cancer recurring in patients after hormonal ablation therapy (7, 8). We have also shown that unlike AD cells, which express high levels of PSA and p21WAF1, AI cells have undetectable levels of both proteins (5).

The role of PSA in prostate cancer is unknown. It is an enzyme belonging to the kallikrein family (9) that is normally found in seminal fluid, but in patients with invasive prostate cancer it can be found in serum, and its level is used as a marker for disease progression or regression (10). In most hormone-refractory patients, PSA expression is maintained even in the absence of androgen. However, in contrast to the AD phase of prostate cancer, serum PSA levels do not necessarily reflect tumor burden in the AI phase of the disease (11). This decrease in the PSA expression with disease progression and a complete loss of PSA expression in a small subgroup of patients has been linked to higher histological grades and more undifferentiated phenotypes (12, 13), but the mechanism of PSA silencing in these tumors has not been elucidated. Our findings may provide the first clue to the mechanism responsible for the loss of PSA expression.

Another protein, p21WAF1, was also found repressed in our AI derivative cell line. p21WAF1 is a member of the cyclin kinase inhibitor protein family, which includes p21, p27, and p57. A low level of p21WAF1 is necessary for the generation of a cdk4/6-cyclin D/p21 complex, but when overexpressed it inhibits the cdk2-cyclin E complex, leading to growth arrest (14) and, under some conditions, to apoptosis (15). The very low level of p21WAF1 in our AI cells may allow them to progress through the cell cycle without the need for androgen stimulation. Mutations of p21WAF1 gene are exceedingly rare in prostate cancer, suggesting other means of regulation. Inhibitors of cyclin-dependent kinases have been shown to be involved in the development of androgen independence of prostate cancer (16–19), although the role of p21WAF1 in this process remains unclear. Forced expression of p21WAF1 in AI cells had inhibitory effects on prostate cancer cell growth (15), but paradoxically patients of which their prostate cancers had overexpressed p21WAF1 had decreased time to progression (20–22). Other studies did not confirm this observation (23), and up to 25–27% of cases in those studies did not express p21WAF1 (20, 21).

Our previous findings showing aberrant regulation of AR, PSA, and p21WAF1 expression in the AI-cells, prompted us to pose the following questions: (a) is the overexpressed AR active in absence of androgen; (b) how can expression of a gene with a functional ARE in its promoter (24), such as PSA, be lost in AI cells; (c) is the high level of activated AR responsible for p21WAF1 suppression; and (d) what is the contribution of the high level of active AR and low level of p21WAF1 to the phenotypic properties of the AI cells, and will a...
reduction in AR level re-establish androgen dependence of growth and sensitivity to induction of apoptosis in these cells.

We were able to show that the overexpression of AR, which happens concomitantly with the acquisition of the AI state in prostate carcinoma cells in culture, is responsible for the silencing of p21\(^{WAF1}\), independence of androgen for growth, and possibly resistance to apoptosis. We propose that reducing AR to a more physiological level may reactivating the phenotypic properties of hormone-sensitive prostate cancer cells in this subgroup of cancers and may make them amenable again to therapy by hormonal ablation and to prevention of apoptosis.

**MATERIALS AND METHODS**

**Reagents.** Wild-type AR-cDNA expression plasmids pAR0 and PSA reporter-plasmid GRE-tk-LUC (GRE) were kindly provided by Dr. Albert O. Brinkmann (Erasmus University Rotterdam, the Netherlands) (25, 26). p21-promoter luciferase reporter construct was a gift from Dr. Lois Silverman (Mount Sinai School of Medicine, New York, NY), and Sp1-p21 reporter (pWPdel-BstXI) was kindly provided by Dr. Yoshiroo Sowa (University of Medicine, Kamigyo-ku, Kyoto, Japan). The full-length (full p21-Luc, Fig. 4) promoter is a 2.4-kb genomic fragment subcloned into the HindIII site of the luciferase reporter vector, pGL2-basic (Promega, Madison, WI; Ref. 27). The pWPdel-BstXI (−133 to +1 relative to the start site) contains all six of the Sp-1 sites but no ARE (28); the Sp1-luc and mtSp1-luc are three tandem repeats of consensus Sp1 sites and mutant Sp1 sites driving luciferase gene (28). Antibodies against PSA, p21, AR, and β-actin as well as secondary antibodies were purchased from DAKO Corp. (Carpinteria, CA) and Santa Cruz Biotechnology, Inc. (Santa Cruz, CA), respectively. Western blotting detection reagents were from Amersham Pharmacia Biotech Inc. Effectene transfection reagent and luciferase assay kit were acquired from Qiagen (Valencia, CA) and Promega. AR-antisense oligonucleotide, 5′-CCACGCTTCTGCACTCCTAC-3′ and its mutant, 5′-CTTCTTCAACTGC-3′ (codon 150–184 of the translation initiation site of exon I) with thioate modifications at each nucleotide, were synthesized by Bio-Synthesis Inc., (Lewisville, TX), 5-Aza, TSA, R1881, and other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO).

**Cell Culture.** LNCaP cells purchased from the American Type Culture Collection (Rockville, MD) were maintained in RPMI 1640 (Life Technologies, Inc., Gaithersburg, MD) with 10% heat-inactivated FBS. The AI LNCaP subline derived from LNCaP cells was maintained in RPMI 1640 containing 10% CSFBS (Hyclone Laboratories, Inc., Logan, UT) and 5 μg/ml of insulin as described previously (5).

MTT was used to estimate cell growth as described previously (29). Briefly, AD or AI cells grown exponentially were aliquoted into 96-well plates at a density of 5000 cells/200 μl well in RPMI 1640 containing 10% CSFBS at 37 °C for 10 nm of R1881. After 24 or 48 h of incubation, 100 μl of the medium was removed from each of the wells, 50 μl of a 1 mg/ml solution of MTT was added to each well, and the cells were incubated for additional 4 h. Two-hundred μl of 0.04 N HCl-isopropanol was added to each well to dissolve the black formazan precipitates, and absorbance at 540 nm was measured on a 96-well microplate reader (Dynex Technologies, Chantilly, VA).

**Western Blotting of Cellular Proteins.** AR, PSA, and p21\(^{WAF1}\) protein levels were determined by Western blot analysis as described previously (30). Total cellular proteins were extracted under conditions described previously (30) from AD or AI cells under various treatments conditions as indicated. Fifty or 100 μg of cellular extracts from LNCaP cells were separated on a 10% SDS-PAGE, electrophoresed to nitrocellulose filters, and immunoblotted with antibodies against p21\(^{WAF1}\), AR, or PSA. The same membranes were stripped and reprobed with β-actin for loading control. Quantitation by densitometry of the X-ray films was done using an Imaging Densitometer Model GS-720 (Bio-Rad, Hercules, CA).

**RT-PCR.** AI cells in exponential phase of growth were treated for 3–7 days with the indicated concentrations of 5-aza. The cells were washed and total RNA extracted with Qiagen RNA isolation kit. An aliquot containing 5 μg of RNA from each treatment was used for the first-strand cDNA synthesis. In each reaction, a 100-μl solution contained 3 μm of random hexamers, 25 mM Tris-HCl, 37 mM KCl, 1.5 mM MgCl\(_2\), 10 mM DTT, 0.25 mM dNTP, 40 units of RNAsin, an RNase inhibitor, and 200 units of reverse transcriptase. The annealing mixture was incubated at room temperature for 15 min, then incubated in a water bath at 41°C for 60 min. The reverse transcriptase enzyme was inactivated by heating the solution to 95°C for 5 min. PCR was then carried out using the Perkin-Elmer PCR reaction kit (Foster City, CA) and PSA primers 5′-GATGACTCCAGCGCAGACT-3′ and 5′-CACAGACCCCCATCTATCCTAC-3′. The PCR reaction was performed using a thermocycler (MJ Research Inc., Watertown, MA) for 30 cycles consisting of denaturation at 94°C for 1 min, annealing at 57°C for 1 min, and extension at 72°C for 2 min. The PCR products were then analyzed on 1.2% agarose gel.

**AR Localization-Immunofluorescence.** AR localization analysis was performed by immunofluorescence using human AR polyclonal antibody from Santa Cruz Biotechnology, Inc. Exponentially growing AD and AI cells were seeded in serum (FBS or CSFBS, respectively) containing medium on glass coverslips precoated with 5 μg/ml of pollylysine and cultured overnight. The medium was replaced with serum-free medium for an additional day and then incubated for 2 or 16 h with or without 10 nm of R1881. The cells were washed, fixed with 3% para-formaldehyde in PBS, blocked with 3% normal goat serum, quenched with 50 mM of NH\(_4\)Cl, permeabilized with 0.1% Triton X-100, washed, and incubated at room temperature for 1 h with 10 μg/ml of anti-AR antibodies in 0.1% PBS/BSA. After washing, the coverslips were incubated with fluorescein-coupled goat antirabbit IgG (1:500 dilution; Sigma Chemical Co.) for 1 h at room temperature, washed, and mounted in Vectashield. The cells were examined in an Axioscope epifluorescence microscope (Zeiss, Oberkochen, Germany) using Plan-Neofluar 40 and 100× (NA 1.5 oil) lenses and digital camera (Diagnostic Instruments) and Photoshop 5 software (Adobe, Mountain View, CA).

**Transfection (Including Antisense Oligonucleotide) and Luciferase Assay.** Exponentially growing AD and AI cells kept in medium with serum were washed once and incubated with serum-free MEM. The cells (60-mm dish) were then transfected with p21-promoter-luciferase reporter. PSA promoter-reporter-plasmid GRE-tk-LUC (GRE), or with pAR0 (to express AR), respectively, all with 1 μg of DNA, and for internal control 0.4 μg of X-tremeGENE Expression Vector pM1-β-Gal (galactosidase gene reporter; Mannheim, Germany) was cotransfected using Effectene according to the manufacturer’s instructions. After transfection (24 h), the cells were exposed for an additional 24 h to increasing concentrations of R1881 or other agents as indicated. For AR antisense oligonucleotide experiments, the medium was replaced with RPMI 1640 containing 10% FBS 5 h after the transfection. The cells were incubated for an additional 43 h then washed and lysed, and the lysates were used for Western blotting and luciferase activity assay using Promega luciferase assay system. For determination of β-galactosidase activity, the cell lysate (15 ml) was incubated in a buffer (0.1 M sodium phosphate (pH 7.5), 2 mM o-nitrophyenyl-galactopyranoside, 10 mM KCl, 1 mM MgCl\(_2\), 0.1% Triton X-100, and 5 mM β-mercaptoethanol) at 37°C for 24 h. The reaction was then terminated with 150 ml of 1 M sodium carbonate, and the absorbancy was measured at 420 nm. The luciferase activities were normalized on the basis of galactosidase activity and were expressed as units/mg of proteins.

**RESULTS**

**Overexpressed AR in AI Cells Is Localized to the Nucleus.** We have shown previously that AD-LNCaP cells, chronically maintained in androgen-poor conditions, gradually progressed to an AI state (5). Concomitantly with the development of the AI state, these cells overexpressed AR and lost the expression of PSA, a well-defined AR target gene. Additional attempts at deriving AI cells through chronic androgen deprivation produced four cell lines with similar properties (results not shown), prompting the question of functionality of the overexpressed AR. To test whether AR was functional, we looked for its relocalization from the cytoplasm to the nucleus in response to androgen treatment. (31–33). As expected, (Fig. 1, top left and third from top, left), in the absence of androgen most of the AR in AD cells was localized to the cytoplasm, and treatment with 10 nm of R1881 for 2 h (Fig. 1, top right) or 16 h (Fig. 1, third from top, right) caused its relocalization to the nucleus, indicating activation. In contrast, in the
AR was localized to the nucleus regardless of R1881 treatment (Fig. 1, second from top, left and right, and fourth from top, left and right). Treatment with R1881 increased somewhat the intensity of fluorescence both in the nuclei and the cytoplasm of AI cells. Moreover, as shown in Fig. 2A, R1881 treatment of both AD and AI cells increased the level of the phosphorylated (activated) protein, additionally suggesting its activation.

Loss of PSA Expression in AI Cells. We tested whether PSA, a known AR target gene, can be induced by R1881 treatment of AI cells, which we showed to have a functional AR. In AD cells used as a positive control, treatment with R1881, which led to an increase in AR level (Fig. 2, A, top panel), produced a concomitant 12-fold up-regulation of the PSA protein (Fig. 2, A, middle panel). However, in the AI cells the level of PSA remained undetectable despite R1881 treatment and a constitutively active, androgen-responsive AR (Figs. 1A and 2A, top panel). Several mechanisms may be responsible for the lack of PSA expression, among them are a mutation or loss of the gene, transcriptional silencing of the gene, or lack of necessary transcription factor(s) or cofactors. To distinguish between these possibilities, we first tested whether R1881 will activate a PSA-promoter in AI cells transfected with a PSA-promoter-luciferase construct; transfected AD cells served as a positive control. The basal expression of the PSA reporter gene in AI cells was 2-fold greater than in AD cells (Fig. 2B), possibly because of the higher levels of AR, and this activity was additionally stimulated by exposure of both cell types to 10 nM of R1881. The AD cells required cotransfection with an AR expression plasmid for full stimulation, possibly because the PSA promoter reporter was competing for transcription factors with the endogenous PSA promoter. In AD cells cotransfected with a plasmid expressing the mutant AR found in LNCaP cells (25, 26), the PSA-reporter gene was similarly induced by R1881 treatment (results not shown). These results showed that R1881 can activate the transcription of a transfected PSA promoter in AI cells, suggesting that it is the endogenous gene that is either silenced or mutated. A common mechanism of gene silencing is hypermethylation of the promoter (34). To test whether this was the case, we incubated AI cells with 5-aza for 3, 5, or 7 days. This treatment is known to inhibit DNA-methylation and cause re-expression of genes silenced by hypermethylation (35). Using RT-PCR detection we showed that 3-day treatment with 5-aza caused a dose-dependent re-expression of the endogenous PSA gene (Fig. 2C), suggesting that the gene is silenced by hypermethylation (5- and 7-day treatment with 5-aza produced a similar induction; results not shown). We do not know whether PSA gene silencing is the result of chronic androgen deprivation or just an
epi-phenomenon of this treatment, but in several additional cell lines derived in a similar way, PSA was also undetected (results not shown). At present, there is no published evidence that the PSA gene is hypermethylated in hormone refractory prostate cancer, but investigation of this possibility in human samples might explain the discrepancy between PSA levels and tumor burden often observed in patients with advanced disease.

Is There a Link between p21<sub>WAF1</sub> and AR Levels? Two additional characteristics of the AI cells derived in our laboratory by chronic androgen deprivation were an undetectable level of p21<sub>WAF1</sub> and resistance to apoptosis (5). Although the loss of p21<sub>WAF1</sub> has not been shown to be universally linked to progression to AI (36), in our hands, four additional attempts at deriving AI cells produced cell lines with a high AR level and a barely detectable (or undetectable) p21<sub>WAF1</sub> expression (results not shown). The loss of p21<sub>WAF1</sub> expression is of interest because of its well-established function as an inhibitor of G<sub>1</sub>-cell cycle progression (37, 38) and regulator of apoptosis (15).

We began to explore the link between the AR and p21<sub>WAF1</sub> in AD cells. We reasoned that if p21<sub>WAF1</sub> functions as an inhibitor of cell cycle progression, R1881 treatment, which stimulates AD cell growth and increases the level and the activation of AR (Fig. 1, A and B), should reduce the p21<sub>WAF1</sub> level. Indeed, we found that in R1881-treated AD cells, the p21<sub>WAF1</sub> protein level was reduced 8-fold compared with an untreated control (Fig. 3A). In contrast, the undetectable level of p21<sub>WAF1</sub> in AI cells was unchanged by this treatment (Fig. 3A).

The observation that R1881 treatment reduces p21<sub>WAF1</sub> protein expression conflicts with a published report showing an induction of p21-promoter activity, p21 mRNA, and protein in LNCaP cells (39, 40). Using the same p21-promoter-reporter (41), we re-examined the effect of R1881 on transiently transfected AD cells. In contrast to the results published previously (39, 40) and in agreement with the reduced level of the endogenous p21<sub>WAF1</sub> protein observed in AD cells treated with R1881 (Fig. 3A), treatment with 1 and 10 nM of R1881 produced a respective 4-fold and 10-fold inhibition of luciferase activity (Fig. 3B). In R1881-treated AI cells, the level of expression of p21<sub>WAF1</sub> promoter activity (Fig. 3B), like the endogenous p21<sub>WAF1</sub> protein, remained undetectable (Fig. 3, A and B). Thus, it is likely that the basal level of p21<sub>WAF1</sub> in AI cells is so low that its additional down-modulation cannot be detected. To additionally examine the role of AR in the down-modulation of p21 expression, we next transfected a breast cancer cell line, MCF-7, with the same promoter-reporter construct. R1881 treatment had no effect on the p21 promoter activity (Fig. 3C) unless the MCF-7 cells were cotransfected with an AR-expressing construct (Fig. 3C) in which case down-regulation was observed. These results indicate that down-regulation of p21<sub>WAF1</sub> is linked to activation of AR and that it is not cell-type specific.

Induction of p21-promoter (luciferase) activity by R1881 treatment of AD cells was shown previously to be partially dependent on specific SP1 sites (39). To identify the response element(s) mediating suppression of p21-promoter activity by R1881, we transfected AD cells either with the p21-promoter-reporter construct or with pWPdel-BstXI plasmid, lacking ARE but containing all six of the SP1 sites (39). As shown (Fig. 3B), whereas the activity of the p21-promoter-reporter was down-regulated by the R1881 treatment in a dose-dependent fashion (Fig. 4A), the treatment did not suppress the activity of a construct lacking the ARE (Fig. 4B). We have also used a construct consisting of three tandem repeats of the SP1 element and a mutated version of this construct and found no effect of R1881 (results not shown). These results suggest that R1881-induced suppression of p21<sub>WAF1</sub> in AD cells requires activation of AR, the presence of ARE in the promoter, and that it is independent of the SP1 elements.

To test the veracity of this conclusion in AI cells, a reversed approach that tested the effect of AR-level reduction on re-expression

![Fig. 3. Effects of R1881 on p21<sub>WAF1</sub> protein expression and promoter activity. A, serum-starved AD and AI cells treated with 10 nM of R1881 for 24 h were harvested and used for determination of p21<sub>WAF1</sub> protein by Western blot (β-actin served as a loading control); B, AD and AI cells in serum-free medium were transfected with 1.0 μg of p21-luciferase-reporter DNA. After the transfection (1 day), the cells were treated with the indicated concentrations of R1881 for 24 h, then washed, lysed, and used to determine luciferase activity; bars, ± SD. C, MCF-7 cells in serum-free medium were transfected with p21-luciferase-reporter or p21-luciferase-reporter and AR expression plasmid. After the transfection (20 h), the cells were exposed to indicated concentrations of R1881 for 24 h, washed, lysed, and used to determine luciferase activity; bars, ± SD. *, P < 0.05; **, P < 0.01.](image1)

![Fig. 4. Dependence of p21<sub>WAF1</sub> suppression by R1881 on intact ARE. AD cells in serum-free medium were transfected with 1 μg/dish of p21-promoter-reporter containing ARE (p21-Luc) DNA (A) or a shorter construct lacking the ARE sequence and containing all six SP-1 sites (pWPdel-BstX1L B). After the transfection (1 day), the cells were exposed for 24 h to different concentrations of R1881 then washed and lysed for the determination of luciferase activity. Data are the means of three separate experiments; bars, ± SD. *, P < 0.05; **, P < 0.01.](image2)
of p21WAF1 was used. To reduce the AR level, AI cells were treated with 0.1 or 0.5 μM of AR antisense oligodeoxynucleotide or with its scrambled form as a negative control and transfected with the p21-promoter-reporter construct. When compared with untreated control or with cells treated with a scrambled version of the oligonucleotide, the antisense reduced the AR level by 50 or 35%, respectively (Fig. 5A). A 50% reduction in AR coincided with a 12-fold increase in the p21-promoter activity and a 35% reduction in AR with a 2.5-fold induction in promoter activity (P < 0.05; Fig. 5B). A slightly greater inhibition of the AR expression and a slightly stronger induction of p21-promoter activity were noted when AI cells were treated with an anti-AR antisense oligonucleotide published previously (As 750/15; Ref. 42) and compared with cells treated with its mutated control (MS750/15; Ref. 42), but the treatment appeared to be more toxic (results not shown). The fact that up-regulation and activation of AR in AD cells inhibits p21WAF1 expression whereas reduction of AR level in AI cells allows the re-expression of p21WAF1 suggests that AR dosage may have a regulatory function in p21WAF1 expression and that loss of p21WAF1 may contribute to the phenotype of advanced prostate carcinoma such as resistance to apoptosis or androgen independence.

Is There a Direct Role for p21WAF1 in Androgen Independence of Growth and Resistance to Apoptosis of AI Cells? To examine directly the contribution of the p21WAF1 protein to the AI cell phenotype we sought ways to induce the expression of this gene. Whereas treatment of AI cells with paclitaxel or navelbine was either ineffective or led to a very slight induction of p21-promoter activity (Fig. 6B, left panel), treatment with TSA, an inhibitor of histone deacetylase, was very effective in inducing p21, confirming results published previously (43). In AD cells, which express high basal level of AR, p21, AND APOPTOSIS IN PROSTATE CANCER CELLS

Fig. 5. Inhibition of AR in AI cells by antisense oligonucleotide: effect on p21WAF1 expression. AI cells in serum-free medium were treated for 48 h with the indicated concentrations of AR antisense oligonucleotide (anti-AR) or its mutated version (anti-mAR) using Effectene. The cells were washed, lysed, and the AR protein level was determined by Western blotting using enhanced chemiluminescence for detection (A) and luciferase activity using Promega luciferase assay kit (B). AR-bands were scanned as in Fig. 5 and normalized to β-actin, which served as a loading control. Data are the mean of three separate experiments; bars, ± SD. *P < 0.05 (compared with the same concentration of mAR oligo).

Fig. 6. Effect of TSA on p21WAF1 expression in AD and AI cells. A, AD and AI cells in exponential growth phase were exposed to the indicated concentrations of TSA for 24 h. The cells were lysed and used to determine p21WAF1 protein content by Western blotting (top) (β-actin served as loading control, middle panel), p21WAF1 bands were scanned as in Fig. 5 and normalized to β-actin (bottom). B, AI cells were transfected in serum-free medium with 1 μg/dish of p21-promoter-reporter DNA. After the transfection (1 day), the cells were treated for 24 h with the indicated compounds (left) or different concentrations of TSA (right) then washed, lysed, and used for luciferase activity determinations. C, AD cells were transfected in serum-free medium with 1 μg DNA/dish of p21-promoter-reporter with intact ARE (p21-Luc) or with Sp1-Luc and msSp1-Luc (see "Materials and Methods") without the ARE sequence. After the transfection (24 h), luciferase activity was measured. D, AI cells in serum-free medium were transfected with 1 μg DNA/dish of p21-Luc (with ARE site). After the transfection (1 day), the cells were exposed for 24 h to TSA in the absence or presence of different concentrations of R1881. The cells were then used to determine luciferase activity; bars, ± SD.
p21\(^{WAF1}\) protein, TSA treatment (0.05–0.4 \(\mu M\)) induced, at the highest concentration, a <2-fold increase of p21\(^{WAF1}\) protein (Fig. 6A, left panel). In contrast, in AI cells with undetectable basal level of p21\(^{WAF1}\) protein, TSA treatment led to a re-expression and a dose-dependent increase in the endogenous p21\(^{WAF1}\) protein (Fig. 6A, right panel). This increase was, at least in part, mediated through transcriptional activation, because a concentration-dependent increase in promoter activity was also observed in AI cells transfected with the p21-promoter-reporter construct and treated with increasing concentrations of TSA. The maximal induction (310-fold) occurred at concentration of 0.5 \(\mu M\) of TSA (Fig. 6B). Concentrations of TSA >0.5 \(\mu M\) produced lesser stimulation of p21, most likely because of toxicity of this compound (Fig. 6B, right panel).

Unlike the inhibition of p21\(^{WAF1}\) by AR, which was ARE and androgen dependent (Figs. 3 and 4), the up-regulation of the p21-promoter activity by TSA was ARE-independent, because the activity of a construct containing only SP1 elements was similarly induced (Fig. 6C). Moreover, activity of a reporter gene driven by a promoter in which the SP1 sites were mutated was not induced by TSA, suggesting that SP1 sites contribute to the TSA effect (Fig. 6C). R1881 treatment that activates AR in these cells was incapable of reducing the high level of p21-promoter activity induced by TSA (Fig. 6D). This suggests that the mechanism of p21\(^{WAF1}\) induction by TSA, which involves acetylation and requires, at a minimum, the presence of an SP1 site, is dissociated from the mechanism by which the high level of AR down-regulates p21\(^{WAF1}\) expression.

Could re-expressed p21\(^{WAF1}\) alter the AI phenotype of these cells? To answer this question, the effect of p21\(^{WAF1}\) re-expression on sensitivity of AI cells to apoptosis induced by a chemotherapeutic agent was tested by treating the cells for two days with 2 and 10 nm of paclitaxel in the absence or presence of TSA. Apoptosis was monitored by formation of a DNA-“ladder.” As shown in Fig. 7, top panel, 10 nm of paclitaxel induced strong apoptosis in AD cells, whereas the AI cells remained completely resistant to this treatment. Only when combined with 10 or 50 nm of TSA, the same concentra-

![Fig. 7. TSA treatment sensitizes AI cells to paclitaxel-induced apoptosis. Exponentially growing AD cells (top panel) and AI cells (bottom panel) were treated with TSA alone, paclitaxel alone, or with a combination of the two drugs for 48 h. The cells were harvested, their DNA extracted, electrophoresed on a 1.2% agarose gel, and stained with ethidium bromide.](Image 345x465 to 524x741)

\[\text{Trichostatin A (nM)}\]
\[\begin{array}{ccccccccc}
0 & 10 & 50 & 0 & 10 & 50 & 10 & 50 & 10 \\
\text{Paclitaxel (nM)} & 0 & 0 & 0 & 2 & 10 & 2 & 10 & 2 \\
\end{array}\]

In AD Cells

In AI Cells

![Fig. 8. Androgen (R1881)-dependence of AI cells treated with AR antisense oligonucleotide. Exponentially growing AI cells were treated with 0.5 \(\mu M\) of AR antisense oligonucleotide or with the mutated oligonucleotide in serum-free medium. (Control cells were mock transfected). After the treatment (1 day), the cells were seeded into 96-well dish at 5000 cells/well in serum-free RPMI 1640 and incubated for 2 days with or without R1881. Cell growth was measured by the MTT method as described in “Materials and Methods.” Bars, ± SD. *, \(P < 0.05\).](Image 50x96 to 290x336)

**DISCUSSION**

Of the biological features of the LNCaP-derived AI cells, most pertinent to the advanced prostate cancer is their ability to grow in the absence of androgen and their resistance to induction of apoptosis. We now show that the overexpression of AR and loss of p21\(^{WAF1}\) expression may contribute to this phenotype. AR overexpression is a common finding in advanced prostate cancer and one that usually
delineates the transition between the success and failure of hormonal ablation therapy. The loss of p21 is not consistently associated with androgen ablation in experimental conditions (36) or in patients (20, 21, 23). Some authors (36) showed that AI LNCaP cells were growth inhibited when exposed to concentrations of R1881 >1 nM and that this treatment induced a persistent activation of another cyclin inhibitor, p27, and a transient induction of p21WAF1. However, every attempt at obtaining an AI derivative in our lab (a total of 5) produced cell lines with an increased AR level and a barely detectable p21WAF1. This fact prompted us to examine whether there was a causal link between the overexpressed AR and p21WAF1 silencing and whether the regulation of these two proteins had an impact on the biological behavior of the AI cells.

Our results indicate that the level of p21WAF1, a regulator of cell cycle progression, appears to be inversely correlated with the level of active AR. This conclusion is supported by the observations that R1881 treatment of AD cells that increases the AR protein level also down-regulates the level of p21WAF1. Inversely, in AI cells, which have a 4-fold greater level of AR and silenced p21WAF1, specific, antisense-induced reduction in AR level leads to an increase in p21WAF1 expression. This inverse relation between AR dosage and p21WAF1 level was found to be true both for the endogenously expressed p21WAF1 protein and p21-promoter activity of a transiently transfected promoter-reporter construct. Although the mechanism through which AR inhibits p21WAF1 expression has not been yet elucidated, our results suggest that it works, at least in part, by inhibiting the promoter activity and that it requires a functional ARE. This effect is somewhat unusual. First, in most cases androgen up-regulates genes, such as PSA, probasin, and others (45). But it was shown before that, depending on the conditions, the same nuclear hormone receptor can function as a repressor or as an activator of transcription (46). Indeed, in a report published previously (39) the activity of a similar ARE-containing p21 promoter transfected into LNCaP cells was induced by R1881 treatment. Moreover, the stimulatory effect was only partially dependent on ARE but was completely eliminated by mutation in one of the Sp1 sites, the Sp1–3 (39). In complete contrast, we found that the down-regulation of p21-promoter activity by R1881 is entirely ARE-dependent and that in absence of ARE, R1881 has no effect regardless of whether the Sp1 elements are wild type or mutant. Although we do not have a good explanation for this discrepancy, we noted that in experiments showing up-regulation of p21WAF1, these authors cultured the AD-LNCaP cells for at least 1 week in charcoal-stripped serum, whereas we kept the cells for 48 h in serum-free medium. Also, growing the LNCaP-AD cell line in androgen-poor conditions, the authors derived an AI cell line with a “normal” level of AR and overexpressed p21WAF1, whereas using apparently similar conditions we derived several individual AI cell lines, all with overexpressed AR and silenced p21WAF1. This suggests that unknown factors may affect the outcome of such derivations and, more importantly, that AR and p21WAF1 expression may not be randomly associated.

Although, the mechanism responsible for the AR overexpression in AI-LNCaP cells remains unknown, it is possible that it involves AR stabilization (6). There is also evidence that mouse and rat AR-promoter contain negative regulatory regions with strong repressor activity (47–49). It remains to be seen whether, after growth in androgen-deprived conditions, a defect in this control mechanism plays any role in the acquisition of the AI phenotype and AR overexpression.

The role of p21WAF1 in the progression of prostate and other cancers remains controversial. Whereas some evidence points to positive effects of its overexpression, because it is linked with more differentiated cancer and better prognosis, other evidence suggests that it may function as an antiapoptotic protein and that its presence may predict for shorter disease-free survival. This confusion may have its base in the importance of the p21 dosage. It has been shown recently (38, 50) that members of the Cip/Kip family of CKIs can function as inhibitors of cyclin-dependent kinases but are also responsible for generation of cyclin d-ckd4 complexes. Under physiological conditions, the cyclin d-ckd4 complex can, without being inhibited, sequester p21WAF1 away from cyclin E-ckd2, allowing ckd2 to function. Only when the p21WAF1 level exceeds the sequestering capacity of cyclin d-ckd4 complex, does it block the exit of cells from G1. Although it remains to be seen whether the same mechanisms function in cancer tissue, the suggestion is that p21WAF1 dosage may have an important effect on cell-cycle progression.

The results of our studies allowed us to test a hypothesis that changing the AR and/or p21WAF1 levels in AI cells to those found in AD cells may lead to a phenotypic mimicry, which may result in functional change such as androgen dependence for growth and sensitivity to apoptosis. Indeed we found that increase in p21 expression, achieved by TSA treatment of AI cells, restored their sensitivity to apoptosis by a chemotherapeutic agent. Although this result fits our prediction, because TSA is not a specific agent and may affect the expression of many genes, it is impossible to attribute this sensitization to p21 overexpression alone. To approach the question more directly we used specific AR-antisense oligos and tested whether a specific reduction in AR dosage, which we showed to induce p21WAF1, will revert the AI cells to androgen dependency. Although difficult to perform, the results of these experiments showed that down-regulation of the AR increases the growth dependence of AI cells on androgen. Interestingly, very recently another group (42) has shown that a complete block (down to 2% of control) of AR expression in AD-LNCaP cells by antisense oligodeoxynucleotides strongly inhibited their growth and induced apoptosis. Using the same antisense oligonucleotides, we showed induction of p21-promoter activity in AI cells (results not shown). More importantly, the growth of AI-LNCaP cells derived by this group, which, like our AI cell line, had a 4-fold higher level of AR than the parental cells, was also growth inhibited by the AR-antisense treatment.

In summary, our results identify the overexpressed AR as an important component of the AI phenotype. In this study we have shown that there is a link between the AR dosage and the level of expression of p21. Restoring a balanced expression of these proteins seems to partially restore the AD phenotype as it pertains to growth dependence on androgen and sensitivity to induction of apoptosis. Because AR is a transcription factor that forms functional complexes with several coactivators and corepressors (51, 52), it is possible that many other pathways, in addition to the one identified here, are at work in AI cells when the receptor is overexpressed. For example, Eder et al. (42) has shown that down-regulation of AR causes down-regulation of epidermal growth factor receptor. This and other examples of AR-induced genes and pathways (1) combine to make a compelling case for considering down-regulation of this receptor as a therapy target for advanced prostate cancer.

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