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Interleukin-6 Regulates Androgen Synthesis in Prostate Cancer Cells

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Abstract

Purpose: The standard systemic treatment for prostate cancer patients is androgen deprivation therapy. Although serum testosterone concentrations were significantly reduced after androgen deprivation therapy, levels of intraprostatic androgens are reproducibly measured at concentrations sufficient to activate androgen receptor and stimulate tumor growth, suggesting that prostate cancer cells may survive androgen deprivation therapies by increasing intracrine androgen synthesis within the prostate. However, factors that regulate *de novo* intracrine androgen synthesis have not been identified. Interleukin-6 (IL-6) has been implicated in the modulation of androgen receptor activation and growth and differentiation in prostate cancer. In this study, we investigate whether IL-6 regulates intraprostatic androgen synthesis in prostate cancer cells.

Experimental Design: Quantitative reverse transcription-PCR and Western blotting were done to detect expression levels of steroidogenic enzymes. AKR1C3 promoter reporter was constructed and analyzed for IL-6–mediated AKR1C3 transcriptional activity. IL-6–mediated signaling was knocked down using small interfering RNA specific to IL-6 receptor and gp130, and the effect on AKR1C3 expression was examined. Intraprostatic androgen levels in prostate cancer cells in culture and in tumors were measured by an enzyme immunoassay (Testosterone EIA kit).

Results: We found that IL-6 increases the expression of genes encoding many steroidogenic enzymes, including *HSD3B2* and *AKR1C3*, involved in androgen biosynthesis. Down-regulation of IL-6 receptor and gp130 expression using specific small interfering RNA abolished IL-6–mediated AKR1C3 expression, suggesting that IL-6 signaling is responsible for AKR1C3 expression. IL-6 increases AKR1C3 promoter activity, indicating that the increase in IL-6–mediated AKR1C3 expression is in part at the transcriptional level. Treatment of IL-6 increased testosterone level in LNCaP cells. The tumor testosterone levels were detected at 378 pg/g in tumors generated from IL-6–overexpressing LNCaP-IL6$^+$ cells inoculated orthotopically into the prostates of castrated male nude mice.

Conclusions: These results suggest that IL-6 increases levels of intracrine androgens through enhanced expression of genes mediating androgen metabolism in prostate cancer cells.

The growth of prostate epithelial cells requires physiologic levels of androgen to both stimulate proliferation and inhibit apoptotic death (1). Because the growth of prostate cancer cells depends on the presence of androgens, almost all patients with advanced prostate cancer respond initially to androgen deprivation and antiandrogen therapy. However, virtually every patient will relapse due to the growth of androgen-independent or castration-resistant cancer cells. Although serum testosterone concentrations are significantly reduced after androgen deprivation therapy, levels of intraprostatic androgens are reproducibly measured to be at concentrations sufficient to activate the androgen receptor (AR) and stimulate tumor growth (2–9). Accumulating evidence suggests that prostate cancer cells may survive androgen deprivation therapies by increasing intracrine androgen synthesis within the prostate.

Intraprostatic androgens can be synthesized *de novo* from cholesterol or other ubiquitous molecular precursors such as dehydroepiandrosterone mediated by genes encoding many steroidogenic enzymes, including *AKR1C3, HSD3B2, SRD5A1, CYP17A1, CYP19A1*, and *UBT2B15*. AKR1C3 (also known as 17βHSD5) converts androstenedione to testosterone in the prostate. HSD3B2 converts dehydroepiandrosterone to androstenedione, a substrate for conversion to testosterone. SRD5A1 converts testosterone to the more potent androgen dihydrotestosterone

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Translational Relevance

One of the greatest challenges facing prostate cancer is its evolution to castration resistance, to which no effective treatment has been developed. Castration-resistant prostate cancer cells often continue to express androgen-responsive genes and often express androgen receptor in the nuclei, suggesting that androgen receptor becomes activated by a mechanism(s) that does not require the exogenous supplies of androgens. Recent findings suggest that the levels of intraprostatic androgens are significantly elevated in prostate cancer. However, factors that regulate intracrine androgen synthesis have not been identified. This study shows for the first time that interleukin-6 (IL-6) regulates the expression of genes encoding many steroidogenic enzymes involved in androgen synthesis. Furthermore, we show that prostate cancer cells synthesize detectable levels of testosterone *in vitro* in the absence of exogenous steroid precursors and IL-6 can increase this process possibly by enhancing the expression of genes encoding steroidogenic enzymes. Therefore, this study provides a rationale for targeting IL-6 signaling in conjunction with androgen deprivation therapy for prostate cancer.

(DHT). It has been speculated that prostate tissues express the full complement of steroid-metabolizing enzymes required for the local synthesis and inactivation of androgens (10). Several recent reports suggested that prostate cancer tissues acquired the ability to synthesize testosterone and DHT from cholesterol, as elevated expression of several steroidogenic enzymes was detected in prostate cancer cells and tissues (5, 11, 12). The expression of these genes involved in regulating androgen metabolism is increased in castration-resistant cancers versus prostate cancers from untreated patients (2, 5–9, 13). These data clearly suggest that testosterone and DHT can be synthesized within prostate tumors through the use of adrenal androgen precursors or through the metabolism of precursors incorporated earlier in the androgen biosynthetic pathway. However, factors that regulate *de novo* intracrine androgen synthesis have not been identified.

Interleukin-6 (IL-6) has been implicated in the modulation of growth and differentiation in many cancers and is associated with poor prognosis in renal cell carcinoma, ovarian cancer, lymphoma, melanoma, and prostate (14). The expression of IL-6 and its receptor has been consistently shown in human prostate cancer cell lines and clinical specimens of prostate cancer and benign prostate hyperplasia (15–17). Multiple studies have shown that IL-6 is elevated in the sera of patients with metastatic prostate cancer and that the levels of IL-6 correlate with tumor burden, serum prostate-specific antigen, and clinically evident metastases (18, 19). In addition to the clinical data that IL-6 is associated with castration-resistant prostate cancer, experimental studies show that IL-6 plays a critical role in prostate cancer cell growth and differentiation. Okamoto et al. (20) showed that IL-6 functions as a paracrine growth factor for the human LNCaP androgen-sensitive prostate cancer cells and an autocrine growth factor for the human DU145 and PC-3 androgen-insensitive prostate cancer cells. It has also been reported that IL-6 mediates LNCaP cell growth arrest and induces neuroendocrine differentiation (21–23). Recently, results from several groups including ours show that IL-6 activates AR-mediated gene expression by activation of the AR through a signal transducer and activator of transcription 3 (Stat3) pathway in LNCaP cells (24–27). Overexpression of IL-6 enhanced prostate-specific antigen mRNA expression in LNCaP cells and can partially rescue LNCaP cells from growth arrest induced by androgen deprivation therapy. In addition, overexpression of IL-6 protects LNCaP cells from undergoing apoptosis induced by androgen deprivation therapy (28). Collectively, these findings suggest that IL-6 can regulate the expression of androgen-responsive genes and promote castration-resistant prostate cancer progression.

In the present study, we investigated the effect of IL-6 on androgen synthesis within the prostate. Our data show that IL-6 regulates intraprostatic androgen synthesis. Prostate cancer cells synthesize detectable levels of testosterone *in vitro* in the absence of exogenous steroid precursors and IL-6 can increase this process possibly by enhancing the expression of genes encoding steroidogenic enzymes.

Materials and Methods

Cell lines and cell culture. LNCaP and CWR22rv1 prostate cancer cells were cultured in RPMI 1640 containing either 10% complete fetal bovine serum (FBS) or 10% charcoal-dextran–stripped FBS and penicillin/streptomycin as described previously (29). LNCaP passage numbers <30 were used throughout the study. IL-6–overexpressing LN-S17 and LNCaP-IL6$^+$ cells were cultured in RPMI 1640 containing 10% FBS as described previously (30, 31). Antibodies against AKR1C3 were purchased from Sigma.

Quantitative reverse transcription-PCR analysis. The levels of mRNA expression of genes encoding steroidogenic enzymes were analyzed by quantitative reverse transcription-PCR (qRT-PCR) with the following primers: AKR1C1/2, 5′-ggtcacttcatgcctgtcct-3′ (forward) and 5′-actctggtcgatgggaattg-3′ (reverse); AKR1C3, 5′-gagaagtaaagctttggaggtca-ca-3′ (forward) and 5′-caacctgctcctcattattgtataaatga-3′ (reverse); HSD3B1, 5′-agaatctagaccactcttctgtccagcttt-3′ (forward) and 5′-ctttgaatt-caactatgtgaaggaatggaa-3′ (reverse); HSD3B2, 5′-cgggcccaactcctacaag-3′ (forward) and 5′-ttttccagaggctcttcttcgt-3′ (reverse); CYP17A1, 5′-gggcggcctcaaatgg-3′ (forward) and 5′-cagcgaaggcgaaggcgataccctta-3′ (reverse); CYP11A1, 5′-agttctcgggacttcgtcagt-3′ (forward) and 5′-ggagcccgccttcttga-3′ (reverse); HSD17B2, 5′-tttgccggagttttgaatgaa-3′ (forward) and 5′-gcaggttcttcgcaattcct-3′ (reverse); HSD17B3, 5′-tggga-cagtgggcagtga-3′ (forward) and 5′-cgagtacgctttcccaattcc-3′ (reverse); RDH5, 5′-gcccgccagcaatgc-3′ (forward) and 5′-cgcccaaagcctgagtca-3′ (reverse); SRD5A1, 5′-acgggcatcggtgcttaat-3′ (forward) and 5′-ccaa-cagtggcataggctttc-3′ (reverse); and CYB5, 5′-cacccgcttcctcaacga-3′ (forward) and 5′-accagcttgttccagcagaac-3′ (reverse). Reactions were conducted with 1 μL RT-PCR cDNA, 0.5 μL each of forward and reverse primers (10 μmol/L), 10.5 μL double-distilled water, and 12.5 μL iQ SYBR Green supermix (Bio-Rad Laboratories). Each reaction was normalized by coamplification of actin. Triplicates of samples were run on the default settings of the Bio-Rad real-time PCR machine (Bio-Rad Laboratories).

Western blot analysis. Cells were lysed in high-salt buffer containing 50 mmol/L HEPES (pH 7.9), 250 mmol/L NaCl, 1 mmol/L EDTA, 1% NP40, 1 mmol/L phenylmethylsulfonyl fluoride, 1 mmol/L sodium vanadate, and 1 mmol/L NaF with the addition of a protease inhibitor cocktail, and total protein was estimated by Coomassie Protein Assay

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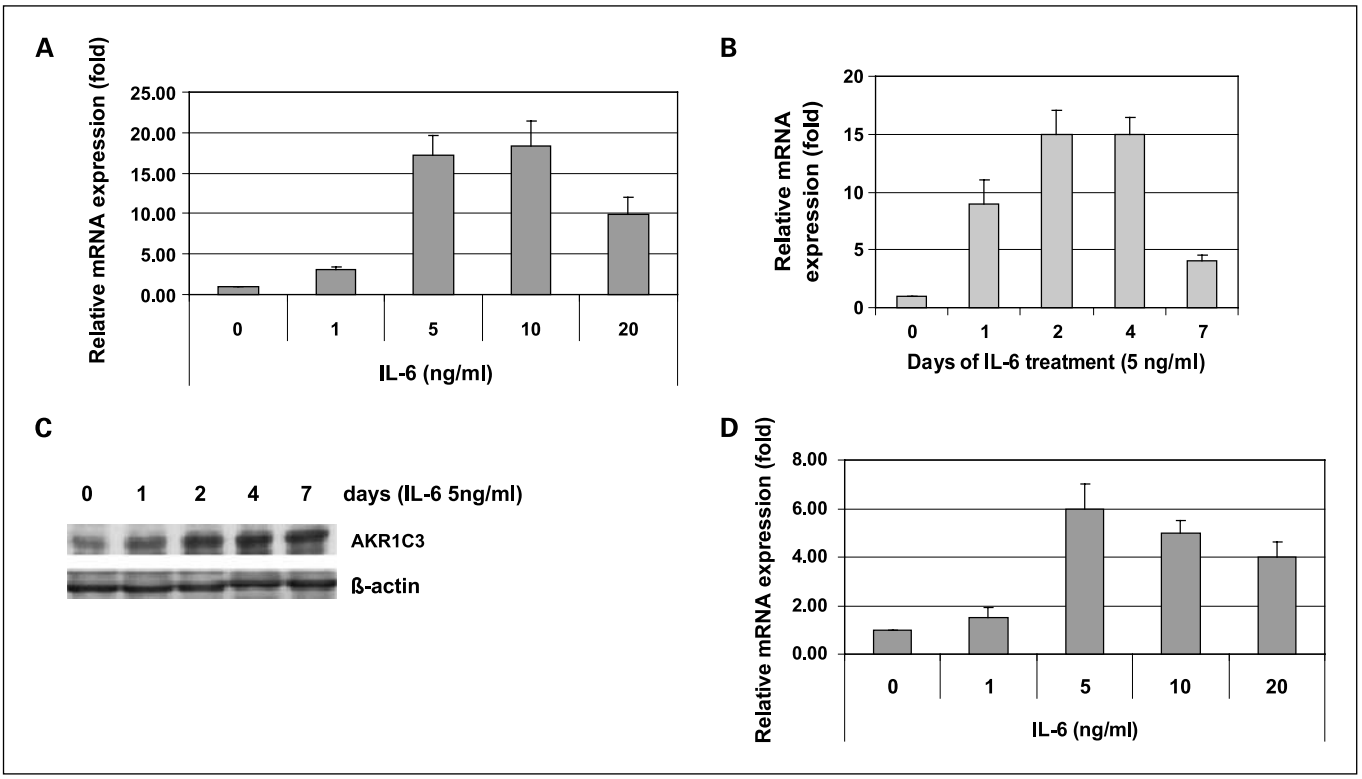

Fig. 1. IL-6 increases AKR1C3 expression. *A,* effect of IL-6 on AKR1C3 mRNA expression in a dose-dependent manner by qRT-PCR analysis. LNCaP cells were treated with IL-6 at different concentrations for 2 d. Total RNA was isolated and subjected to qRT-PCR analysis. *B,* effect of IL-6 on AKR1C3 mRNA expression by qRT-PCR analysis. LNCaP cells were treated with 5 ng/mL IL-6 for different time points. Total RNA was isolated and subjected to qRT-PCR analysis. *C,* AKR1C3 protein expression by Western blot analysis. LNCaP cells were treated with 5 ng/mL IL-6 for different time points. Whole-cell lysates were subjected to Western blot analysis using antibodies against AKR1C3 protein. β-Actin was used as protein loading control. *D,* effect of IL-6 on AKR1C3 mRNA expression in CWR22rv1 cells. CWR22rv1 cells were treated with IL-6 at different concentrations for 2 d. Total RNA was isolated and subjected to qRT-PCR analysis.

Reagent (Pierce). Equal amounts of protein were loaded on 10% SDS-PAGE and transferred to nitrocellulose membranes. The membranes were blocked with 5% nonfat milk in 1× PBS + 0.1% Tween 20 and probed with the indicated primary antibodies. The chemiluminescent signal was detected by enhanced chemiluminescence kit (Amersham) after incubation with the appropriate horseradish peroxidase–conjugated secondary antibodies.

Human AKR1C3 promoter luciferase reporter gene constructs. A 2,170-bp human AKR1C3 promoter was amplified from genomic DNA isolated from human LNCaP prostate cancer cells using primers spanning 5′-flanking regions of *AKR1C3* gene (forward, 5′-tggcttgatggtgtcctaca-3′; reverse, 5′-caacccaatacgggtttcac-3′). The amplified fragment was subsequently subcloned into a firefly luciferase expression vector pGL4-Luc (Promega). The authenticity of this construct pGL4-AKR1C3-promoter was confirmed by matching its sequence against human AKR1C3 promoter.

Transient transfection and luciferase reporter assay. Cells were seeded in 24-well plates (5×10^4 per well) and grown to 70% to 80% confluence and transiently transfected using transfection reagent Tfx-20 (Promega). pGL4-AKR1C3-promoter luciferase construct was cotransfected with pRL-TK (TK promoter-*Renilla* luciferase construct as internal control). Briefly, pGL4-AKR1C3-promoter luciferase construct and pRL-TK were mixed in 200 μL serum-free RPMI 1640 in a 1.5 mL tube for each well of 24-well plates. After incubation at room temperature for 30 min, the DNA/Tfx mixture was added to each well. The DNA-Tfx complex was replaced by complete medium containing 10% FBS following incubation for 1 h. The luciferase activity was determined 24 to 48 h after transfection using a dual-luciferase reporter assay system (Promega). Cell lysates (25 μL/well) were used for measurement of luciferase activity in a luminometer by first mixing the cell lysates (25 μL) with 20 μL luciferase assay reagent for measuring firefly luciferase activity and subsequently adding 20 μL Stop-Glo reagent for measuring *Renilla* luciferase activity. Data were normalized to *Renilla* luciferase activity (internal control) as arbitrary units.

Testosterone analysis by enzyme immunoassay. Steroid extractions were done as described by Locke et al. (32) with modifications. Briefly, frozen tumors were homogenized and extracted twice with ethyl acetate (1:1, v/v) and dried down using a centrivap centrifugal evaporation system. Samples were then reconstituted in 100 μL enzyme immunoassay (EIA) buffer and used for testosterone measurement using Testosterone EIA kit (Cayman Chemical). This assay is based on the competition between testosterone and a testosterone-acetylcholinesterase conjugate (testosterone tracer) for a limited amount of testosterone antiserum. The detection limit of this assay is 6 pg/mL. The specificity of this assay is 100% for testosterone and 27% for 5α-DHT. The testosterone EIA tests were done according to the manufacturer's instructions. Briefly, 50 μL each of sample, testosterone-acetylcholinesterase tracer, and testosterone antiserum were added to each well. The plates were washed to remove any unbound reagents and developed by adding Ellman's reagent (which contains the substrate to acetylcholinesterase). Samples were tested in triplicates. Accurate sample concentrations of testosterone were determined by comparing their respective absorbance values with those obtained for the reference standards plotted on a standard curve.

Statistical analysis. Data are shown as mean ± SD. Multiple group comparison was done by one-way ANOVA followed by the Scheffe procedure for comparison of means. $P < 0.05$ was considered statistically significant.

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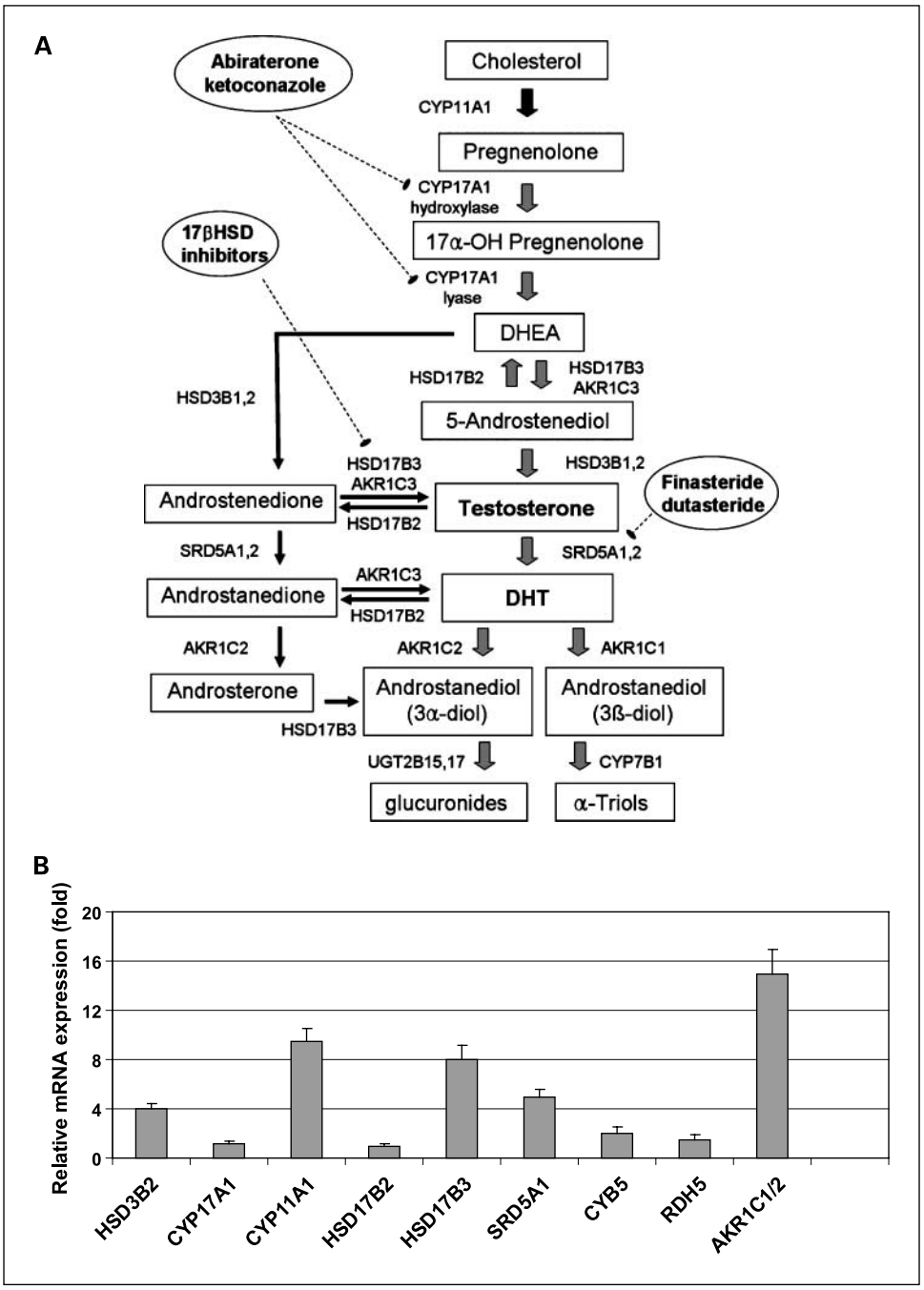

Fig. 2. IL-6 affects the expression of genes encoding steroidogenic enzymes. *A*, schematic outline of the steroidogenic pathway from cholesterol to DHT. Broken lines, enzymes targeted by inhibitors. *B*, qRT-PCR analysis of nine genes encoding steroidogenic enzymes involved in androgen synthesis. LNCaP cells were treated with 5 ng/mL IL-6 for 2 d. Total RNA was isolated and subjected to qRT-PCR analysis. The levels of expression are reported as relative mRNA expression (fold) to the control without IL-6 treatment.

Results

IL-6 increases AKR1C1-3 gene expression. One highly significant recent development concerning castration-resistant prostate cancer is the discovery that levels of intracellular androgens and the expression of enzymes involved in androgen biosynthesis, such as HSD3B1/2 and AKR1C1-3, are up-regulated in castration-resistant prostate cancer (2, 13). HSD3B1/2 and AKR1C1-3 are significantly elevated in castration-resistant prostate cancer specimens compared with benign prostate cancer (13). However, factors that regulate *de novo* intracrine androgen synthesis have not been identified. To evaluate whether IL-6 affects *de novo* androgen synthesis, we examined the effect of IL-6 on the expression of genes encoding steroidogenic enzymes, including AKR1C1-3, involved in androgen biosynthesis. Real-time qRT-PCR analysis showed that IL-6 increased AKR1C3 mRNA expression in a dose-dependent manner in LNCaP prostate cancer cells (Fig. 1A). The levels of AKR1C3 mRNA were increased 3-fold at 1 ng/mL IL-6 and 17-fold at 5 ng/mL IL-6 and maximized to ~19-fold at 10 ng/mL IL-6 after 2 days of treatment (Fig. 1A). The optimal time for maximal induction of AKR1C3 mRNA expression in LNCaP cells was ~2 days after addition of IL-6 and decreased to ~4-fold at 7 days of treatment (Fig. 1B). Consistent with the induction of AKR1C3 mRNA expression, IL-6 increased the levels of AKR1C3 protein expression as shown by Western blot analysis (Fig. 1C). We also

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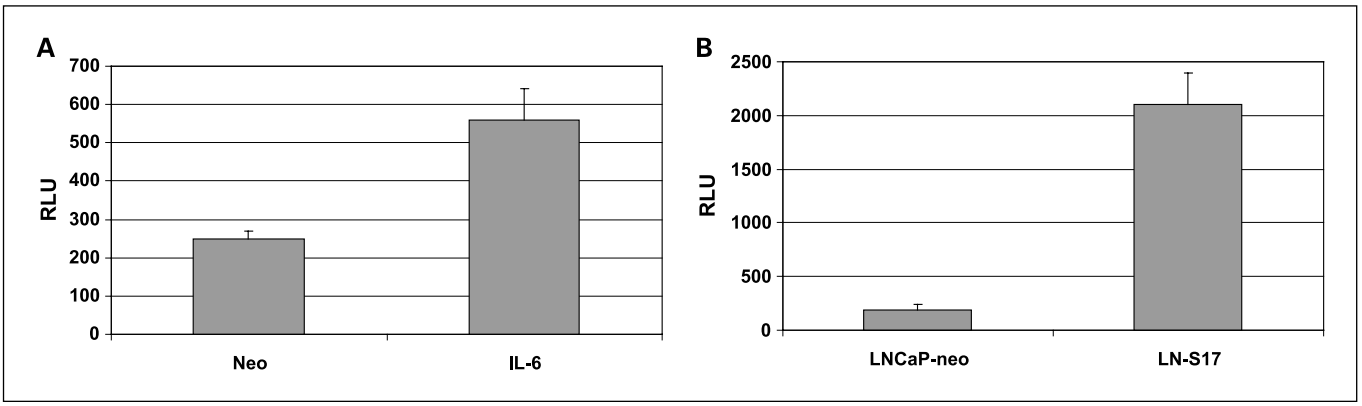

Fig. 3. IL-6 activates AKR1C3 transcription. *A,* LNCaP cells were cotransfected with pcDNA3.1-IL6 (500 ng) or empty vector pcDNA3.1 (500 ng) and pGL4-AKR1C3-Luc reporter construct (200 ng). Luciferase activity was measured 2 d after transfection and normalized to *Renilla* luciferase activity (internal control) as arbitrary units. *B,* pGL4-AKR1C3-Luc reporter construct (200 ng) was transfected to LNCaP-neo and IL-6–overexpressing LN-S17 cells. Luciferase activity was measured 2 d after transfection and normalized to *Renilla* luciferase activity (internal control) as arbitrary units.

examined the effects of IL-6 on AKR1C3 mRNA expression in CWR22rv1 prostate cancer cells by qRT-PCR analyses and found that IL-6 increased AKR1C3 mRNA level by 6-fold at a concentration of 5 ng/mL (Fig. 1D), suggesting that the effect of IL-6 on genes encoding steroidogenic enzymes is not LNCaP cell specific. Collectively, these results showed that IL-6 increases AKR1C3 gene expression in human prostate cancer cells.

Effect of IL-6 on the expression of other steroidogenic enzymes. Biosynthesis and metabolism of testosterone from cholesterol require several key components of genes encoding steroidogenic enzymes, including CYP17A1, CYP11A1, HSD17B2, HSD17B3, HSD17B5, SRD5A1, RDH5α, CYB5, and HSD3B2 (Fig. 2A). In addition to induction of expression of AKR1C3, we examined the effects of IL-6 on other genes encoding steroidogenic enzymes in LNCaP cells by real-time RT-PCR analysis. The expression of HSD3B2, CYP11A1, HSD17B3, SRD5A1, and AKR1C1/2 at mRNA level is significantly elevated ($P < 0.05$) by IL-6, whereas CYP17A1, HSD17B2, CYB5A, and RDH5α at mRNA level show no significant change by IL-6 treatment (Fig. 2B).

IL-6 up-regulates AKR1C3 promoter activity. Because IL-6 increased AKR1C3 mRNA expression, we next examined the effects of IL-6 on AKR1C3 promoter activity. We amplified a 2,170-bp 5′-flanking sequence of *AKR1C3* gene from genomic DNA isolated from LNCaP cells and cloned it into a firefly luciferase expression vector pGL4-basic. The amplified 2,170-bp 5′-flanking region of *AKR1C3* gene was sequence confirmed. To examine whether pGL4-AKR1C3-promoter luciferase plasmid can be transcriptionally regulated by IL-6, we tested the effect of overexpression of IL-6 on AKR1C3 promoter activity. Coexpression of IL-6 with the 2,170-bp pGL4-AKR1C3-promoter luciferase reporter plasmid in LNCaP cells showed a 2-fold increase in the promoter activity (Fig. 3A). To determine the effect of constitutive overexpression of IL-6 on AKR1C3 transcriptional activity, we transfected pGL4-AKR1C3 luciferase reporter into LN-S17 cells, a cell line constitutively overexpressing IL-6 established previously by ectopically expressing IL-6 through introduction of a full-length IL-6 cDNA into LNCaP cells. The AKR1C3-luc activity was significantly higher in IL-6–overexpressing LN-S17 compared with the parental LNCaP cells (Fig. 3B).

Knockdown of IL-6 signaling blocked IL-6–mediated AKR1C3 gene expression. IL-6 signaling is through its receptor composed of an IL-6–specific receptor subunit (α chain) and a signal transducer, gp130 (β chain; ref. 33). To examine whether IL-6–mediated AKR1C3 gene expression in LNCaP cells requires

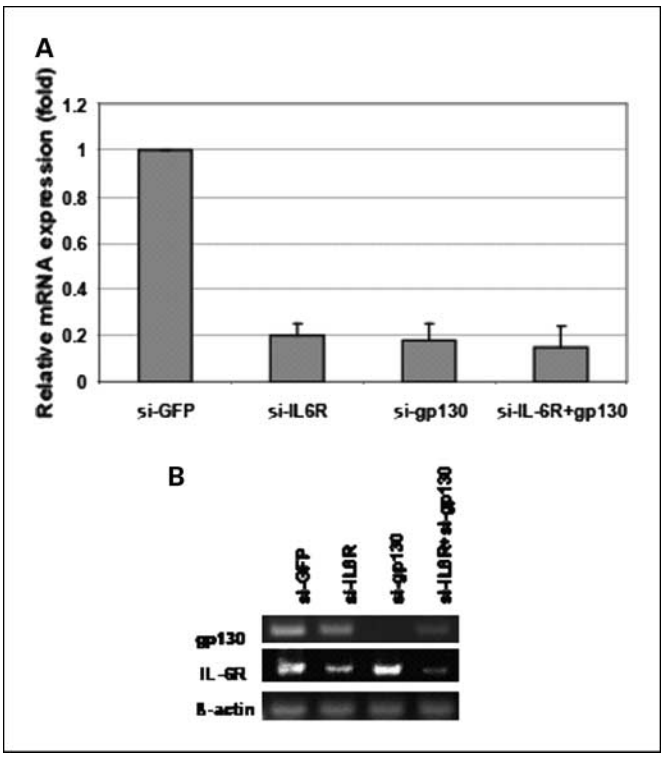

Fig. 4. Knockdown of IL-6 signaling blocked IL-6–mediated AKR1C3 transcription. LNCaP cells were transfected with small interfering RNA specific to IL-6 receptor, gp130, or combination of IL-6 receptor and gp130. Green fluorescent protein (*GFP*) small interfering RNA was used as control. The cells were switched to medium containing 5 ng/mL IL-6 after transfection and continued in culture for 2 d. *A,* total RNA was isolated and subjected to qRT-PCR analysis of AKR1C3 mRNA expression. *B,* expression of gp130 and IL-6 receptor mRNA was determined by RT-PCR.

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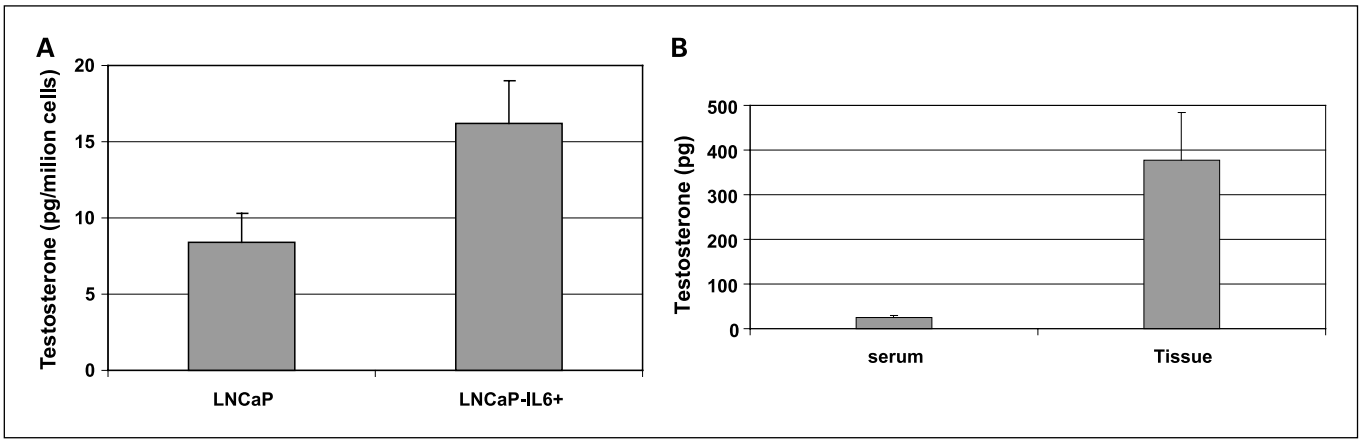

Fig. 5. Treatment with IL-6 increases testosterone level in LNCaP cells. *A,* LNCaP and LNCaP-IL6$^+$ cells were cultured in serum-free medium for 48 h and cell lysates were isolated and subjected to determination of testosterone by EIA. The levels of testosterone in LNCaP-IL6$^+$ cells are significantly higher than LNCaP cells ($P < 0.05$) *B,* testosterone levels in prostate tumors from LNCaP-IL6$^+$ cells orthotopically implanted into castrated male nude mice. LNCaP-IL6$^+$ cells were orthotopically injected into the prostate of male nude mice. The mice were castrated 3 d after injection. The mice were sacrificed after 4 mo, and tumors were collected and used for testosterone determination by EIA. Columns, mean from four mice; bars, SD.

IL-6 signaling, we knocked down IL-6 receptor and its signal-transducer gp130 expression, respectively, or together using their specific small interfering RNA. Knockdown of IL-6 receptor and gp130 expression significantly blocked IL-6–induced AKR1C1/2 and AKR1C3 mRNA expression (Fig. 4). These results suggest that knockdown of IL-6 signaling blocks IL-6–induced steroidogenic enzyme expression involved in androgen biosynthesis.

Treatment with IL-6 increases testosterone in LNCaP cells. LNCaP-IL6$^+$ cells were generated previously by culturing LNCaP continuously in medium containing 5 ng/mL IL-6 over a long period (31). Long-term treatment with IL-6 promotes LNCaP cell growth *in vitro* by an autocrine mechanism accompanied by activation of AR signaling (31). Growth of high passages (>58 passages) of LNCaP cells treated with IL-6 (LNCaP-IL6$^+$) was accelerated. These cells can grow well in androgen-depleted charcoal-stripped FBS conditions (34). To determine whether these cells synthesize androgens, parental LNCaP and LNCaP-IL6$^+$ cells were cultured in serum-free medium for 48 hours and cell lysates were isolated and used for determination of testosterone by EIA. As shown in Fig. 5A, the levels of total testosterone were detected in cell lysates from both LNCaP (8.2 pg/million) and LNCaP-IL6$^+$ (16.1 pg/million) cells. The levels of total testosterone in LNCaP-IL6$^+$ cells were ~2-fold higher than LNCaP cells (Fig. 5A). These data suggest that LNCaP cells synthesize detectable levels of testosterone *in vitro* in the absence of exogenous steroid precursors and IL-6 treatment can increase this process possibly by enhancing the expression of genes encoding steroidogenic enzymes.

Levels of androgens in LNCaP-IL6$^+$ tumors. To determine whether LNCaP-IL6$^+$ cells synthesize testosterone *in vivo*, LNCaP-IL6$^+$ cells were inoculated orthotopically into the prostates of castrated male nude mice (protocol approved by the Institutional Animal Care and Use Committee). Mice were sacrificed after 4 months and tumors were collected. To measure intraprostatic androgen levels, androgens were quantified in the tumors using EIA. The tumor testosterone levels were detected at 378 ± 105 pg/g tissue in LNCaP-IL6$^+$ tumors (Fig. 5B). The serum testosterone levels were detected at 25 ± 11 pg/mL.

Discussion

Castration-resistant prostate cancer cells often continue to express androgen-responsive genes, such as *prostate-specific antigen*, and often express AR in the nuclei (35, 36), suggesting that AR becomes activated by a mechanism(s) that does not require the exogenous supplies of testicular level of androgen. Possible mechanisms by which prostate cancer cells could become castration resistant include the following. First, mutations or amplification of the *AR* gene could increase tumor cell sensitivity to very low levels of androgen or could allow it to respond to other steroids or even antiandrogens (37, 38). Second, alterations of the interactions between AR and some of its coregulators could allow wild-type or mutated AR to become activated by other steroids or antiandrogens (39). Third, AR can be sensitized by growth factors and cytokines (24, 25, 30, 40, 41). In addition, clonal expansion of cells with neuroendocrine differentiation may also be involved in castration-resistant prostate cancer progression (42). Recent evidence showed that prostate cancer cells may survive androgen deprivation therapies by regulating intracrine androgen synthesis through the use of cholesterol or earlier precursors within prostatic tumor. However, factors that regulate *de novo* intracrine androgen synthesis have not been identified. In this study, we examined the effects of IL-6 on intraprostatic androgen synthesis and showed that IL-6 increases the expression of genes encoding steroidogenic enzymes, including HSD3B1/2 and AKR1C1-3, resulting in an increase in the levels of androgens in the prostate.

Androgens play a critical role in the pathogenesis of both benign and malignant prostate cancer. One highly significant recent development concerning castration-resistant prostate cancer is the discovery that levels of intracellular androgens and the expression of enzymes involved in androgen biosynthesis, such as HSD3B1/2 and AKR1C1-3, are up-regulated in castration-resistant prostate cancer (2, 5–9, 13). HSD3B1/2 and AKR1C1-3 are significantly elevated in castration-resistant prostate cancer specimens compared with benign prostate cancer (5–9, 13). This was confirmed by our experimental xenograft

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prostate cancer model in which LNCaP-IL6$^+$ cells were implanted orthotopically into the prostates of the castrated male mice. The serum testosterone levels were significantly reduced to 25 pg/mL after castration. However, the levels of testosterone in the LNCaP-IL6$^+$ tumors grown in the prostates of castrated mice were as high as 378 pg/g. DHT concentrations as low as 2.9×10^{-3} pg/g have been shown to transactivate AR in prostate cancer cell lines (43); therefore, the levels observed in LNCaP-IL6$^+$ tumors would seem sufficient for AR activation. The much higher levels of testosterone in the prostate tumor (378 pg/g) compared with the lower levels of testosterone in the serum (25 pg/mL) suggest that LNCaP-IL6$^+$ tumors develop compensatory mechanisms by synthesizing intratumoral androgens, possibly by use of cholesterol or precursors to synthesize androgens through IL-6–mediated elevation of steroidogenic enzymes, including HSD3B2 and AKR1C1-3, as suggested by the observation using castration-resistant prostate cancer LNCaP tumor models (32).

Our study is consistent with others that LNCaP cells contain all enzymes necessary for testosterone and DHT synthesis from cholesterol or other ubiquitous molecular precursors such as dehydroepiandrosterone (12, 44). DHT, the predominant androgen in the prostate, is synthesized from testicular testosterone by prostatic 5α-reductase. DHT can also be synthesized from androstenedione (4-dione) by a two-step reduction reaction, in which 5α-reductase converts androstenedione to 5α-androstane-3,17-dione, which is then converted to DHT via 17-β-hydroxysteroid dehydrogenase type 5 (AKR1C3 or HSD17B5) in a reversible reaction (45). AKR1C3 is also a member of the aldo ketoreductase superfamily. This enzyme catalyzes the conversion of androstenedione to DHT and is expressed in the liver, prostate, breast, adrenals, endometrium, mammary gland, and ovary. In contrast to other members of the AKR1C family, AKR1C3 predominantly functions as a reductive enzyme and converts 4-dione to T and has the potential to be a major peripheral source of androgens in the prostate. AKR1C3 overexpression has been observed in leukemia and cancers of the prostate, breast, endometrium, and head and neck (46). A variant allele of AKR1C3 decreases the risk of lung and prostate cancers (47). Our data show that IL-6 increases the levels of AKR1C3 mRNA and protein expression in both LNCaP and CWR22rv1 cells, suggesting that IL-6 regulates this process. Furthermore, IL-6 increases the levels of AKR1C3 mRNA expression in LNCaP-IL6$^+$ cells by ~8-fold at 5 ng/mL IL-6 (data not shown), suggesting that IL-6 can further enhance AKR1C3 expression in cells expressing endogenous IL-6. In addition to the up-regulation of AKR1C3 expression by IL-6, other genes encoding steroidogenic enzymes are also regulated at the transcriptional level by IL-6, including up-regulation of AKR1C1/2, HSD3B2, CYP11A1, HSD17B3, and SRD5A1. These enzymes are involved in the conversion of cholesterol to dehydroepiandrosterone (CYP11A1), catalyzation of the transformation of 4-dione into testosterone (HSD17B3 and AKR1C3), and conversion of testosterone to the higher-affinity DHT (SRD5A1). These data suggest that IL-6 regulates androgen synthesis at multiple steps by regulating the expression of enzymes participating in androgen metabolism.

We have cloned AKR1C3 promoter containing ~2.2 kb upstream 5′-flanking sequences of *AKR1C3* gene and showed that IL-6 up-regulates AKR1C3 promoter activity. Sequence analysis indicated several consensus binding sites of Stat3 and nuclear factor-κB within this region, indicating that IL-6 downstream signaling such as Stat3 may be involved in IL-6–mediated AKR1C3 transcriptional regulation. We are currently investigating the role of Stat3 in regulating AKR1C3 expression.

Androgen and IL-6 interactions are complicated. IL-6 obviously potentiates the action of androgen; however, androgen may limit the action of IL-6 by inducing suppressors of cytokine signaling (48). In summary, in addition to activation of AR, increasing cell proliferation and differentiation, inducing prostate cancer cell neuroendocrine differentiation, and protecting cells from apoptotic cell death, IL-6 may regulate intraprostatic androgen synthesis. Our results show that prostate cancer cells synthesize detectable levels of testosterone *in vitro* in the absence of exogenous steroid precursors and IL-6 can increase this process possibly by enhancing the expression of genes encoding steroidogenic enzymes.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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