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13. Abstract ***(Maximum 200 Words)*** *(abstract should contain no proprietary or confidential information)*

In the current work we are exploring the hypothesis that interleukin-6 (IL-6) contributes to the progression of prostate cancer (PCA) through enhancing AR activity. *Objective I is to determine the mechanism through which IL-6 sensitizes AR to androgen.* We have determined that IL-6 induces AR expression through stimulation of the AR gene promoter. Furthermore, IL-6 enhances AR activity in the absence of androgen. *Objective II is to evaluate if inhibition of IL-6 diminishes PCA proliferation in a rodent model.* We have determined that administration of anti-IL-6 antibody induces regression of established PC-3 PCA cell line tumors in a murine xenograft model through induction of apoptosis. These results indicate that IL-6 may contribute to the progression of PCA to an apparent androgen-independent state, through either inducing the AR in the absence of androgens, or sensitizing AR to the low levels of androgens typically present in men undergoing androgen deprivation therapy. Additionally, our results suggest that inhibition of IL-6 activity may promote regression of PCA. Taken together, these data indicate that targeting IL-6 in PCA patients may help diminish the progression of this disease.

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INTRODUCTION:

Prostate carcinoma (PCA) initially responds to androgen deprivation. However, it usually reoccurs in a form that is unresponsive to further hormonal manipulations. This latter form of PCA, termed androgen independent cancer, inexorably progresses resulting in the demise of the patient. The mechanism responsible for development of androgen independent cancer is unknown. However, some clues may be found in the response of PCA cells to the cytokine interleukin-6 (IL-6). Specifically, IL-6 and IL-6 receptor are expressed in PCA. Furthermore, inhibition of IL-6 in prostate cell culture diminishes PCA cell proliferation demonstrating the presence of an autocrine mechanism of IL-6 activity. Finally, IL-6 has been shown to both activate the androgen receptor (AR) in the absence of androgen and sensitize the AR to androgen. These observations have important implications regarding androgen-deprivation therapy. In the current work we are exploring the hypothesis that IL-6 contributes to the progression of PCA, that is observed post-androgen deprivation, through enhancing AR activity. We will test our hypothesis by the following combination of *in vitro* and *in vivo* objectives: *Objective I: Determine the mechanism through which IL-6 sensitizes AR to androgen. Objective II: Evaluate if inhibition of IL-6 diminishes PCA proliferation in a rodent model. Objective III: Determine if IL-6 contributes to PCA progression post-androgen deprivation* In summary, these experiments should help identify the extent and mechanism of IL-6's role in PCA progression. They are designed to elucidate if IL-6 promotes androgen hyperresponsive tumors or truly androgen-independent tumors. These data should provide a rationale for target IL-6 for inhibiting PCA progression.

BODY:

Statement of Work Tasks for the Initial Funding Period:

Task 1. Determine the mechanism through which IL-6 sensitizes AR to androgen. (months 1-18)

- perform Western and PCR analyses to determine if IL-6 increases AR expression (months 1-3)
- perform transfection experiments to determine if IL-6 increases AR gene activation (months 4-9)
- perform transfection experiments to determine if IL-6 increases AR transactivation strength (months 10-12)
- perform bandshift assays to determine if IL-6 increases nuclear levels of AR (months 13-14)

These aims have all been accomplished and are presented in Appended Manuscript #1 (1). In brief, we have demonstrated that IL-6 induces AR activity and expression through activation of the AR gene promoter. This results in increased AR nuclear translocation and results in both androgen-independent activation of the AR and increased sensitivity of AR to androgen.

Task 2. Produce reagents needed for Tasks 3 and 4 (months 1-12)

- prepare anti-murine IL-6 and anti-murine isotype antibodies for Tasks 3 and 4 by inoculating mice with hybridoma, collecting ascites fluid, purifying antibodies (months 1-4)
- maintain tumor in nude mice until ready for transplantation [20 mice] (months 1-12)

We were able to work with a collaborator to obtain the necessary anti-IL-6 antibody; thus, these aims have been accomplished. In summary, we were able to use an anti-human IL-6 antibody alone, as opposed to anti-murine, because murine IL-6 did not cross-react with human IL-6 receptor. Use of these antibodies is reported in Appended Manuscript #2 (2).

Task 3. Evaluate if inhibition of IL-6 diminishes PCA proliferation in a rodent model [80 mice] (months 10-21)

- initiate tumor model in sham operated or orchiectomized nude mice and administer IL-6 and isotype antibody (months 10-16)
- euthanize mice, analyze tumor tissue for growth, AR/IL-6 expression and androgen sensitivity (months 17-21)

We have performed an in vivo study using anti-IL-6 to inhibit prostate tumor growth. These results are reported in appended Manuscript #2 (2). In summary, we have demonstrated for the first time that inhibition of IL-6 results in diminished prostate cancer tumor growth in an animal model. Furthermore, we demonstrated that inhibition of IL-6 was associated with induction of apoptosis in the tumors. These results have important implications for the role of IL-6 in clinical prostate cancer. We have summarized the importance of IL-6 in PCA in a review article (3).

KEY RESEARCH ACCOMPLISHMENTS:

- IL-6 activates AR in the absence of androgen
- IL-6 sensitizes the AR to androgen
- IL-6 increases AR levels through activation of the AR
- Inhibition of IL-6 diminishes PCA growth in vivo
- Inhibition of IL-6 promotes apoptosis of PCA cells in vivo.

REPORTABLE OUTCOMES:

1. MANUSCRIPTS
    a. Lin DL, Whitney MC, Yao Z, and Keller ET. Interleukin-6 induces androgen responsiveness in prostate cancer cells through up-regulation of androgen receptor expression. Clin Cancer Res. 7:1773-81, 2001.
    b. Smith PC and Keller ET. Anti-interleukin-6 monoclonal antibody induces regression of human prostate cancer xenografts in nude mice. Prostate, 48:47-53, 2001.
    c. Smith PC, Hobisch A, Lin DL, Culig Z, Keller ET. Interleukin-6 and prostate cancer progression. Cytokine Growth Factor Reviews. 12:33-40, 2001.
2. ABSTRACTS
    a. Smith PS and Keller ET. Anti-Interleukin-6 Monoclonal Antibody Inhibits Prostate Cancer Growth In Vivo. American Association for Cancer Research Annual Meeting, New Orleans, 2001.

CONCLUSIONS:

The results suggest that IL-6 is contributes to the pathophysiology of prostate cancer. In combination with previously published reports that IL-6 is associated with prostate cancer morbidity suggests that targeting IL-6 may promoter regression and/or prevent progression of prostate cancer in patients. Additionally, these results imply that IL-6 may be a putative biomarker of prostate cancer. This latter hypothesis will demand some attention in future studies.

REFERENCES:

1. Lin D., Whitney M., Zhi Y., Keller E. 2001. Interleukin-6 induces androgen responsiveness in prostate cancer cells through upreguation of androgen expression. *Clin Cancer Res*;**7**:1773-1781.
2. Smith P.C., Keller E.T. 2001. Anti-interleukin-6 monoclonal antibody induces regression of human prostate cancer xenografts in nude mice. *Prostate*;**48**:47-53.
3. Smith P.C., Hobish A., Lin D., Culig Z., Keller E.T. 2001. Interleukin-6 and prostate cancer progression. *Cytokine Growth Factor Rev*;**12**:33-40.

Interleukin-6 Induces Androgen Responsiveness in Prostate Cancer Cells through Up-Regulation of Androgen Receptor Expression[1]

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ABSTRACT

Interleukin-6 (IL-6) induces prostate cancer (CaP) cell proliferation *in vitro*. Several lines of evidence suggest that IL-6 may promote CaP progression through induction of an androgen response. In this work, we explored whether IL-6 induces androgen responsiveness through modulation of androgen receptor (AR) expression. We found that in the absence of androgen, IL-6 increased prostate-specific antigen (PSA) mRNA levels and activated several androgen-responsive promoters, but not the non-androgen responsive promoters in LNCaP cells. Bicalutamide, an antiandrogen, abolished the IL-6 effect and IL-6 could not activate the PSA and murine mammary tumor virus reporters in AR-negative DU-145 and PC3 cells. These data indicate the IL-6 induces an androgen response in CaP cells through the AR. Pretreatment of LNCaP cells with SB202190, PD98059, or tyrphostin AG879 [p38 mitogen-activated protein kinase (MAPK), MAP/extracellular signal-regulated protein kinase kinase 1/2, and ErbB2 MAPK inhibitors, respectively) but not wortmannin (PI3-kinase inhibitor) blocked IL-6-mediated induction of the PSA promoter, which demonstrates that IL-6 activity is dependent on a MAPK pathway. Finally, IL-6 activated the *AR* gene promoter, resulting in increased AR mRNA and protein levels in LNCaP cells. These results demonstrate that IL-6 induces AR expression and are the first report of cytokine-mediated induction of the *AR* promoter. Taken together, our results suggest that IL-6 induces AR activity through both increasing AR gene expression and activating the AR in the absence of androgen in CaP cells. These results provide a mechanism through which IL-6 may contribute to the development of androgen-independent CaP.

INTRODUCTION

When initially identified, most CaP[3] require androgenic stimulation for growth. After androgen withdrawal, most prostate cells undergo an active process of programmed cell death (1). Unfortunately, after an initial response to androgen deprivation therapy, CaP usually recurs in a form that grows independent of androgen and is unresponsive to further androgen withdrawal (2). The mechanism responsible for development of androgen independent cancer is unknown. However, accumulating evidence suggests that the androgen-independent phenotype results when CaP cells acquire a paracrine or autocrine growth mechanism through production of growth factors and cytokines (3–5).

A putative growth factor that promotes prostate cancer growth is IL-6. Initially identified as a cytokine that exhibits pleiotropic functions, IL-6 regulates gene expression in a number of different organs, modulates immune function, stimulates the hypothalamic-pituitary axis, promotes osteoclast resorption of bone, and stimulates bone marrow (reviewed in Ref. 6). It has been shown to induce acute-phase proteins and a number of immediate early genes, including *jun B*, circulating intercellular adhesion molecule 1 (*ICAM-1*), and IFN regulatory factor 1 (*IRF1*; Refs. 7–9). The biological activities of IL-6 are mediated by the IL-6 receptor, which binds IL-6 specifically and with low affinity, and gp130, which associates with the IL-6-IL-6 receptor complex, resulting in high-affinity binding and activation of intracellular signaling. Evidence has accumulated that suggests IL-6 may be an important autocrine and/or paracrine growth factor for CaP (4, 5, 10, 11).

AR, an essential mediator of androgen action, is a ligand-dependent transcription factor belonging to the nuclear steroid hormone receptor superfamily (12). The receptor contains a ligand (androgen)-binding domain and a DNA-binding domain. In the absence of androgens, the AR stays mainly in the cytoplasm in an inactive form. When the AR is activated by androgen, it binds to an enhancer ARE in the regulatory region of target genes as the key step for promoter activation (13). During the progression of CaP, AR expression becomes heterogeneous (14).

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[3] The abbreviations used are: CaP, prostate carcinoma(s); AR, androgen receptor; ARE, androgen responsive element; BIC, bicalutamide; DHT, dihydrotestosterone; ER, estrogen receptor; IL-6, interleukin-6; lux, luciferase; MMTV, murine mammary tumor virus; NFDM, nonfat dried milk; PSA, prostate-specific antigen; RT-PCR, reverse transcription-PCR; SLP, sex-limited protein; PI, phosphatidylinositol; MAP, mitogen-activated protein; MAPK, MAP kinase; MEK, MAPK kinase; EGF, epidermal growth factor; GFP, green fluorescent protein; STAT, signal transducer and activator of transcription; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

LNCaP is an androgen-responsive human CaP cell line, derived from a lymph node CaP metastasis (15). LNCaP cells have AR mutations and have been shown to be sensitive to antiandrogens and estrogen as well as to androgens (16, 17). IL-6 has been shown to confer androgen-like activity on LNCaP cells (18, 19). Although the ability of IL-6 to induced androgen-like activity has been associated with activation of several signal transduction cascades (19, 20), the mechanism through which IL-6 induces AR activity is unknown. Accordingly, in the current report, we explore the mechanism through which IL-6 induces an androgen response in prostate cancer cells.

MATERIALS AND METHODS

Cell Lines and Reagents. LNCaP, PC-3, and DU-145 prostate cancer cell lines were purchased from the American Type Culture Collection (Rockville, MD). These cell lines have been previously shown by several investigators to express both the IL-6 receptor α and the IL-6 receptor β (gp130) chains (4, 5, 21). The cells were maintained in RPMI 1640 (Life Technologies, Inc., Gaithersburg, MD) supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 0.1 mg/ml streptomycin at 37°C in a 5% CO_2 incubator. Recombinant human IL-6 was obtained from Sigma Chemical Co., Inc. (St. Louis, MO). Rabbit antihuman AR polyclonal antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz Biotechnology, CA). The antiandrogen BIC (Casodex, 20 μM; Zeneca Pharmaceuticals; Macclesfield, United Kingdom) was provided by Dr. K. Olsen (University of Michigan, Ann Arbor, MI). The following reagents: BSB202190 (5 μM), PD98059 (15 μM), wortmannin (100 nM), and tyrphostin AG879 (10 μM) were purchased from Calbiochem, San Diego, CA. The PSA reporter, p1.5kPSA-Lux, containing the proximal −1500-bp of the PSA promoter driving the lux cDNA was a gift from Dr. P. Hsiao (NIEAS, Durham, NC). The MMTV (22) reporter, pMMTV-Lux, containing 150 bp of the MMTV promoter driving the lux cDNA has been described previously (23). The mouse SLP (24) reporter, pc′Δ9(slp)-Lux, containing 900 bp of the SLP promoter driving the lux cDNA is a gift from Dr. D. Robins (University of Michigan, Ann Arbor). The plasmid pPAI(−800/+22)-Lux containing a human type-1 plasminogen activator inhibitor gene promoter driving the lux cDNA was a gift from Dr. T. Gelehrter (University of Michigan, Ann Arbor). The plasmid ERE-tk-Lux, containing tandem ER response elements upstream of the thymidine kinase promoter driving the lux cDNA, was a gift from Dr. R. Koenig (University of Michigan, Ann Arbor).

Semiquantitative RT-PCR. Semiquantitative RT-PCR was performed as previously described, with minor modification (18). Briefly, total RNA was isolated from cells using TRIzol method (Life Technologies, Inc.). Using Superscript one-step RT-PCR system (Life Technologies, Inc.), we subjected 1 μg of total RNA to thermal cycling as follows: one cycle at 50°C for 30 min; at 94°C for 2 min with an additional 29 cycles at 94°C for 15 s; at 56°C for 30 s; and at 72°C for 1 min, and 5 min at 72°C for the final extension. PSA and β2-microglobulin primer sequences were: PSA 418/21 sense, 5′-GGCAGGTGCTTG-TAGCCTCTC-3′; PSA 939/21 antisense, 5′-CACCCGAGCAG-GTGCTTTTGC-3′; β2-microglobulin, sense, 5′-ATGCCTGC-CGTGTGAACCATGT-3′; and β2-microglobulin antisense 5′-AGAGCTACCTGTGGAGCAACCT-3′ as previously described (18). The AR primers were designed to flank the ligand-binding domain: sense, 5′-ACACATTGAAGGCTATGAATGTC-3′; and antisense, 5′-TCACTGGGTGTGGAAATAGATGGG-3′. For quantitation, either PSA or AR primers (10 μM, 1 μl) was mixed with β2-microglobulin (5 μM, 1 μl) for RT-PCR. The PCR products were then resolved in the 1.3% agarose gel and bands were analyzed with ChemiImager v3.3 software (Alpha Innotech, San Leandro, CA). Target fragment levels were normalized against β2-microglobulin, and data are presented as target mRNA:β2-microglobulin ratio.

Transient Transfection. Transfection was conducted using SuperFect (Qiagen, Valencia, CA) as recommended by the manufacturer. Briefly, LNCaP cells were plated in 6-well plates at a confluency of 60–70% 24 h before transfection in medium supplemented with fetal bovine serum or in medium supplemented with charcoal-stripped serum as indicated (See Fig. 1). Cells in 1 ml of medium in 6-well plates was incubated at 37°C with 2 μg of plasmid DNA (1 μg of reporter, internal control 50 ng of pRL-cytomegalovirus, and 950 ng pBluescript) mixed in 10 μl of SuperFect. After the 2 h incubation, 1 ml of fresh medium was added to the cells. The medium was replaced the next day, and the cells were incubated for an additional 24 h. Total protein was then collected by lysis buffer and lux activities were measured by using Dual-Luciferase System (Promega, Madison, WI) and captured by TD-20/20 Luminometer (Turner Designs, Sunnyvale, CA).

Nuclear Lysate Preparation. Nuclear protein extract from LNCaP cells was prepared as described previously (23). Briefly, cells were harvested after being washed twice in PBS buffer. For nuclei preparation, cells were resuspended in hypotonic buffer [10 mM HEPES-KOH (pH 7.9), 1.5 mM $MgCl_2$, 10 mM KCl, and 0.1% NP40] and incubated on ice for 10 min. Nuclei were precipitated with 3000 × *g* centrifugation at 4°C for 10 min. After washing once with hypotonic buffer, the nuclei were lysed in the lysis buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 1% Triton X-100) and incubated on ice for 30 min. The nuclear lysates were precleared by 20,000 × *g* centrifugation at 4°C for 15 min. Protein concentration was measured by Bradford assays.

Western Blot Analysis. LNCaP cells were cultured for 2 days in media with charcoal stripped serum. The cells were then stimulated for 24 h with the indicated concentrations of IL-6 and then lysed by multiple freeze thawing in 0.25 M Tris buffer. Western blot analysis was performed as previously described (23) with rabbit anti-AR (N-20, Santa Cruz Biotechnology).

Detection of AR by Indirect Immunofluorescence and Fluorescence Imaging. LNCaP cells were grown on glass coverslips for immunofluorescence. The coverslips were rinse once in PBS and fixed with 4% paraformaldehyde in PBS pH 7.4 for 10 min. Neutralization took place for 5 min in 50 mM NH_4Cl in PBS. The coverslips were then washed twice in PBS. Cells were incubated for 15 min with 1% BSA, NFDM, 0.3% Triton X-100 in PBS. The coverslips were incubated for 1 h in rabbit anti-AR (N-20) diluted 1:100 in 1% BSA, 5% NFDM, and 0.1% Tween 20 in PBS. The slips were then extensively washed in PBS-Tween-20 and incubated for 1 h with goat antirabbit IgG conjugated with FITC (Santa Cruz Biotechnolo-

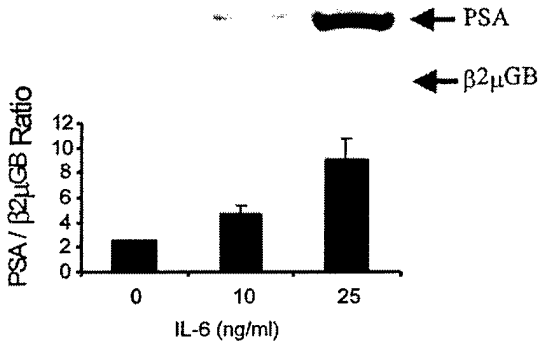


Fig. 1 IL-6 induces PSA mRNA levels from LNCaP cells in the absence of androgen. LNCaP cells were plated to a 60% confluence in charcoal-stripped media in 100-mm plates. Cells were then treated with 0, 10, or 25 ng/ml IL-6 for 24 h. After incubation, total RNA was collected and evaluated for the PSA mRNA levels by semiquantitative RT-PCR. PCR products were subjected to electrophoresis and measured by densitometry. The mRNA levels are expressed as the ratio of PSA: β2-microglobulin. The gel shown here is the result of a typical experiment. Results are shown as mean (± SE) from three independent experiments.

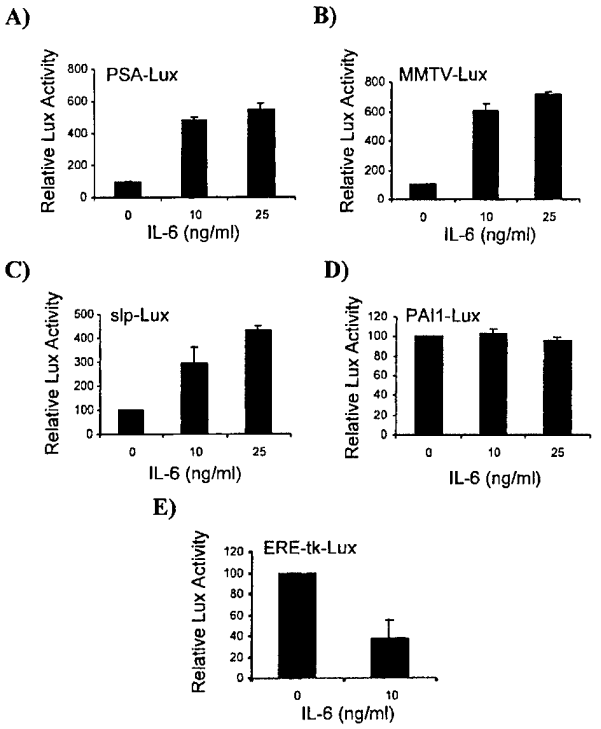


Fig. 2 IL-6 activates androgen-responsive promoters in the absence of androgen in LNCaP cells *in vitro*. LNCaP cells were cotransfected with pRL-CMV (for normalization using the dual lux assay) and one of the following: (*A*) p1.5kPSA-Lux, containing the proximal −1.5-k bp of the PSA promoter); (*B*) pMMTV-Lux, containing 150 bp of the MMTV promoter; (*C*) pc′Δ9(slp)-Lux, containing −900 bp of the SLP promoter; (*D*) pPAI-1-Lux, containing a human type-1 plasminogen-activator-inhibitor gene promoter (−800/+22); or (*E*) pERE-tk-Lux, containing tandem repeat of estrogen response elements upstream of the thymidine kinase promoter. Cells were then incubated in charcoal-stripped medium with the addition of 0, 10, or 25 ng/ml IL-6 for 24 h. After incubation, lux levels were measured using a luminometer. Data are reported as the mean (±SE) lux activity relative to the basal activity, which was set as 100%, from three independent experiments.

gy), diluted 1:500 in 1% BSA, 5% NFDM, and 0.1% Tween 20 in PBS. The images were captured using a fluorescent microscope. A GFP-AR expression plasmid (a gift from Dr. M. Lu, Harvard University, Boston, MA) was transfected into PC3 cells. The fusion fluorescent protein was imaged by confocal microscopy.

RESULTS

IL-6 modulates both cell growth (5, 21, 25, 26) and the androgen regulatory signal pathway in LNCaP cells (18, 19). However, results regarding IL-6 expression and activity in LNCaP cells are rather inconsistent (27). To determine whether IL-6 induces an androgen-like response in the LNCaP model system in our hands, the ability of increasing doses of IL-6 to modulate steady-state mRNA expression of the androgen-responsive PSA (28) in LNCaP cells was determined. In the absence of androgens, IL-6 caused a dose-dependent increase of PSA mRNA levels (Fig. 1). The mean PSA mRNA levels increased ~2- and 3.5-fold at 10 and 25 ng/ml of IL-6, respectively. Thus, IL-6 increases steady-state mRNA expression of an androgen-responsive gene, which is consistent with the possibility that it may activate the AR.

To determine whether IL-6 increased steady state mRNA levels of androgen-responsive genes through increasing their transcription, the ability of IL-6 to activate that PSA promoter was determined. IL-6 induced more than a 4-fold increase of PSA promoter activity (Fig. 2*A*). To next evaluate whether IL-6-mediated transactivation extended to other androgen-responsive promoters, we measured the ability of IL-6 to activate the MMTV(22) and the mouse SLP (24) promoters. IL-6 (10–25 ng/ml) transactivated both the MMTV and SLP promoters approximately 6-fold and 3- to 4-fold, respectively (Fig. 2*B* and 2*C*) demonstrating that IL-6 can induce a variety of androgen-responsive genes. To determine whether IL-6-mediated transactivation was a nonspecific phenomenon, we evaluated the ability of IL-6 to induce several androgen nonresponsive genes; the human type-1 plasminogen activator inhibitor gene promoter and the thymidine kinase (tk) promoter downstream of a tandem ER response elements. In contrast to the androgen-responsive genes, these promoters were not activated by IL-6 (Fig. 2*D* and 2*E*). Taken together, these data suggest that IL-6 transactivates androgen-responsive genes with some degree of specificity.

The observation that IL-6 activated several different androgen-responsive genes suggests that there is a common mechanism through which IL-6 mediates this effect. A candidate target for IL-6-mediated transactivation of androgen-responsive promoters is the AR. Thus, to test if IL-6 transactivates androgen-responsive promoters through the AR, we incubated the cells with BIC (Casodex), an antiandrogen that is able to completely abolish AR function (29). BIC completely blocked the IL-6-mediated induction of the PSA and MMTV promoters (Fig. 3*A*) suggesting that IL-6 induces these genes through the AR. To further support IL-6's requirement for AR, the ability of

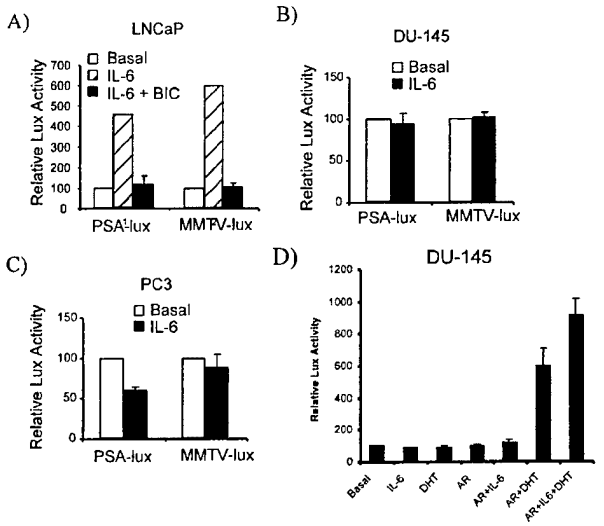


Fig. 3 IL-6-induced transcriptional activity requires an active AR. (*A*) LNCaP cells, (*B*) DU-145 cells, and (*C*) PC3 cells were cotransfected with pRL-CMV (for normalization using the dual lux assay) and either p1.5kPSA-Lux or pMMTV-Lux. The cells were then untreated (*Basal*) or treated with either IL-6 (10 ng/ml) or IL-6 (10ng/ml) and 20 μM BIC for 24 h as indicated. After incubation, lux levels were measured using a luminometer. *D*, DU-145 cells were cotransfected with pRL-CMV and p1.5kPSA-Lux. *Lanes 4–7*, cells were additionally cotransfected with 50 ng of pCMV-AR (*AR*). Cells were then incubated in charcoal-stripped media with the addition of 10 ng/ml IL-6, or 10 nM DHT, or both (as indicated) for 24 h. Data are reported as the mean (±SE) lux activity relative to the basal activity, which was set as 100%, from three independent experiments.

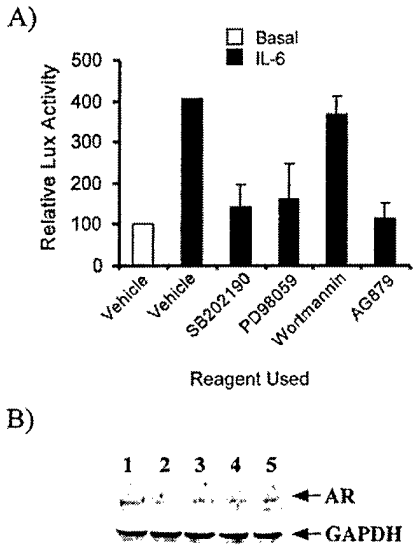


Fig. 4 PSA promoter induction by IL-6 was blocked by specific inhibitors to signal transducers. *A*, LNCaP cells were cotransfected with pRL-CMV (for normalization using the dual lux assay) and p1.5kPSA-Lux. The cells were then incubated with vehicle, SB202190 (5 μM), PD98059 (15 μM), wortmannin (100 nM), or tyrphostin AG879 (10 μM) for 30 min prior to IL-6 administration (10 ng/ml) for 24 h. After incubation, lux levels were measured using a luminometer. Data are reported as the mean (±SE) lux activity relative to the basal activity, which was set as 100%, from three independent experiments. *B*, the immunoblot of AR and GAPDH from 40 μg of cell lysate. LNCaP cells were cultured in medium containing IL-6 (*Lane 1*) or IL-6 plus pretreatment of the inhibitor reagents described above (*Lanes 2–5*, respectively).

IL-6 to activate the PSA and MMTV promoters in two AR-negative CaP cell lines that express the IL-6 receptor α and β (gp 130) chains (4, 5, 21), DU-145 and PC3, was determined. IL-6 did not induce PSA or MMTV promoter activity in these cell lines (Fig. 3*B* and 3*C*). When AR was transiently expressed in DU-145 cells, IL-6 alone still did not induce the PSA promoter (Fig. 3*D*, *Lane 5*) although DHT alone did (Fig. 3*D*, *Lane 6*). However, IL-6 increased the androgen-induced PSA promoter activity (Fig. 3*D*, *Lane 7*). Similar results were obtained for activation of the MMTV promoter (data not shown). Collectively, these data suggest that AR is required, but not sufficient, for the IL-6-mediated activation of androgen-responsive promoters.

Several nonsteroidal substances induce AR activity through a variety of signal transduction pathways (18, 30–34). IL-6 activates PI-3 kinase pathway in several cell types including LNCaP cells (25, 26, 35, 36). Furthermore, IL-6 requires the growth factor receptors ErbB2 and -3 for signaling in LNCaP cells (20). To determine whether IL-6-induced androgen-responsive gene activation is mediated through these signal transduction pathways, the ability of kinase inhibitors to abrogate IL-6-mediated activation of the PSA promoter was evaluated. SB202190 (a p38 MAPK inhibitor), PD98059 (a MEK1/2 inhibitor), and tyrphostin AG879 (a ErbB2 MAPK inhibitor) but not wortmannin (a PI-3-kinase inhibitor), blocked IL-6-mediated induction of the PSA promoter activity (Fig. 4*A*). LY294002 (a PI-3-kinase inhibitor) has been shown to cause program cell death to LNCaP (37). To ensure that the negative result that we obtained in Fig. 4*A* was not derived from cell death, we examined the cell viability by trypan blue exclusion on cells that followed the same treatment. We found no significant variation of the living cell number among different treatment group (data not shown). Because, in the report of Carsen *et al.*, addition of growth factor or serum in addition to LY294002 protected the LNCaP cells from apoptosis, it is plausible that the addition of IL-6 after cells were given the inhibitors may save cells from the cytotoxic effect. Also, to ensure that this response was not attributable to kinase inhibitor-mediated alteration of AR levels, we measured AR protein in the cells. The level of AR from cells treated with IL-6 and kinase inhibitors was the same as to that of IL-6 alone (Fig. 4*B*), except for a slight reduction of AR level from cells treated with SB202190 (Fig. 4*B*, *Lane 2*). Because MAPK inhibition blocked IL-6-mediated activation of the PSA promoter, we next assessed whether activation of MAPK could induce PSA promoter expression. Accordingly, we treated cells with EGF (a MAPK activator known to activate ER-dependent promoter activities; Ref. 38). EGF (100 ng/ml) did not induce PSA promoter activity in either the absence or the presence of transgenic AR in DU145 cells (data not shown). However, EGF (100 ng/ml) induced ERE-tk-Lux activity by 30-fold in the presence of transgenic ERα, which demonstrated that EGF was functional (data not shown). Together, these data suggest that MAPK activation is

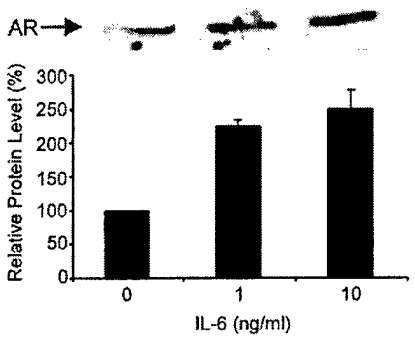


Fig. 5 Total AR levels from LNCaP cells are up-regulated by IL-6. LNCaP cells were treated with 0, 1, or 10 ng/ml of IL-6 for 24 h in charcoal-stripped media. Total cellular protein was then collected and subjected to Western blot analysis (200 μg/lane) using an antihuman AR NH_2-terminal antibody. Bands were measured by densitometry. The blot shown here is the result of a typical experiment. Data are reported as mean (±SE) percentage of AR protein level relative to untreated cells, which was set as 100%, from three independent experiments.

necessary, but not sufficient, for IL-6-mediated activation of the PSA promoter.

Hobisch *et. al.* reported that IL-6 induces LNCaP cell androgen responsiveness in the presence of low doses of androgen (18). One mechanism, through which IL-6 may increase androgen responsiveness in an AR-dependent fashion, as the current data demonstrate, is through induction of AR expression. Thus, the ability of IL-6 to increase AR expression in LNCaP cells was determined. The average AR protein amount doubled in the presence of 1 ng/ml of IL-6, whereas a 2.5-fold increase of mean AR level was observed when the IL-6 concentration was increased an order of magnitude (Fig. 5, *Lanes 2* and *3*). These data are consistent with the possibility that IL-6 sensitizes LNCaP cells to androgen through increased AR expression. However, the AR typically translocates to the nucleus to exert its function on gene expression (39). Accordingly, we determined whether IL-6 induces nuclear AR translocation. In the absence of DHT or IL-6, AR was detected primarily in the cytoplasm (Fig. 6, *A* and *D*). IL-6 induced a nuclear AR pattern similar to that of DHT-induced nuclear AR expression (compare Fig. 6, *B*, *C*, *E*, and *F*). In agreement with the immunofluorescent staining and the GFP tagging results, IL-6 increased AR levels in nuclear extract from LNCaP cells (Fig. 6*G*). Taken together, these data suggest that IL-6 induces androgen-responsiveness through increasing total AR protein levels that results in increased nuclear AR.

It is plausible that IL-6 induces AR expression through transcriptional or translational mechanisms. To determine whether IL-6 increases AR expression levels through transcriptional mechanisms, the ability of IL-6 to modulate AR mRNA levels was initially determined. IL-6 at 10 and 25 ng/ml increased steady-state AR mRNA levels 3-fold and 4-fold, respectively (Fig. 7). To evaluate whether IL-6 increases steady-state AR mRNA levels through transcription, the ability of IL-6 to activate the AR promoter was determined. IL-6 activated the AR promoter in a dose-dependent fashion (Fig. 8). Specifically, IL-6 at 5 and 25 ng/ml induced 3- and 3.5-fold activation, respectively, of the AR promoter (Fig. 8, *Lanes 2* and *3* compared with *Lane 1*). These data correlated well with the magnitude of increased steady-state AR mRNA levels induced by IL-6. To determine whether the IL-6-induced increase of AR levels is sufficient to increase androgen activity in the absence of ligand, we overexpressed AR in LNCaP cells (Fig. 9, *Lanes 2* and *3*). Surprisingly, overexpression of AR diminished basal PSA mRNA levels (Fig. 9). These results demonstrate that increased AR levels alone are not sufficient to mediate IL-6 induction of androgen response in the absence of androgen, which suggests that IL-6 activates the AR in addition to increasing AR expression.

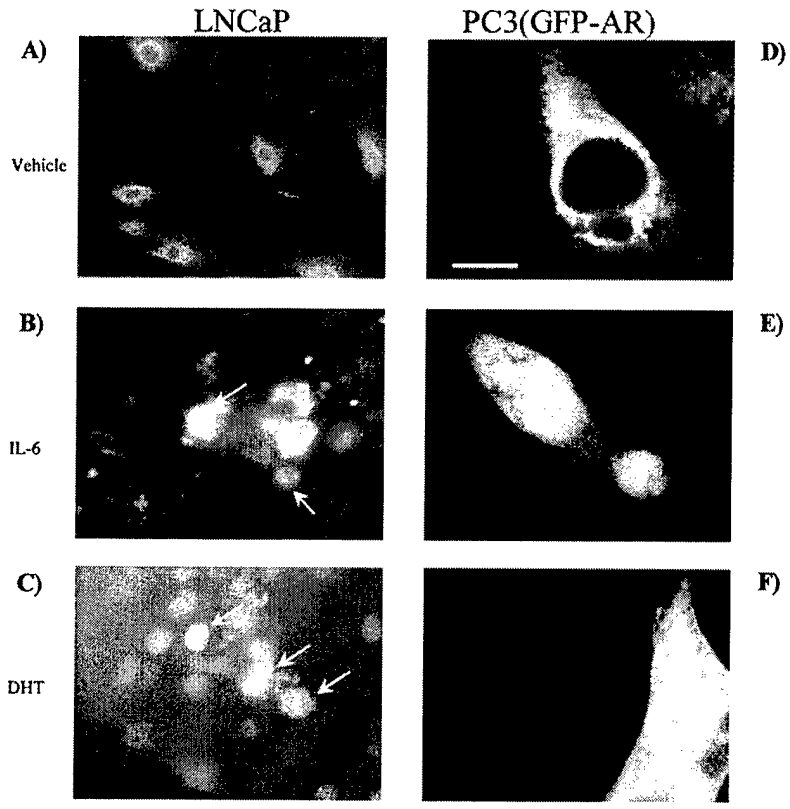
DISCUSSION

Induction of AR gene transcription is a relatively understudied area. Prior to the current study, only androgens were reported to activate the AR gene promoter. Thus, the present report is the first description of a non-androgen inducer of AR gene transcription, namely IL-6. In addition to increasing AR gene expression, this study demonstrated that IL-6 activates the AR in the absence of androgen. The ability of IL-6 to activate AR requires MAPK activity; however, MAPK alone is not sufficient to activate the AR. Taken together, these results suggest that IL-6 may promote androgen-independent prostate cancer progression through both increasing AR levels and increasing AR activity.

Elevated serum IL-6 expression is associated with the morbidity and progression of prostate cancer (40). Furthermore, IL-6 induces prostate cancer cell proliferation and protects prostate cancer cell lines from chemotherapeutics *in vitro* (41, 42). Our finding that IL-6 induced three different androgen-responsive promoters (PSA promoter, MMTV promoter, and SLP promoter) is consistent with a previous report that IL-6 activated an ARE-driven minimal promoter reporter vector (18). Our study extended this previous report by evaluating the affect of IL-6 on the ARE in the context of several natural androgen-responsive promoters, thus approximating the natural promoter activity better than isolated AREs. Our observations suggest that one mechanism through which IL-6 may contribute to prostate cancer progression is the ability to activate a general androgen response in prostate tumors. Such a response could be associated with increased tumor proliferation in the absence of androgens, which would contribute to the development of androgen independence.

The observation that IL-6 activated several androgen-responsive promoters but not androgen-nonresponsive promoters suggests that IL-6 has a degree of specificity for inducing androgen-like response. One potential mechanism that could account for such a response is the induction of AR activity. The observations that BIC blocked the effect of IL-6 and that IL-6 could not activate the PSA and MMTV promoters in AR-negative CaP cells support this hypothesis. Two non-mutually exclusive mechanisms that could account for the ability of IL-6 to increase AR action are increased AR levels and increased AR function.

The observation that IL-6 induced androgen-responsive genes in the absence of androgens through an AR-dependent mechanism suggested that IL-6 activates AR function. This



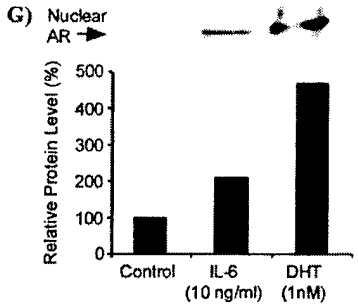


Fig. 6 IL-6 increases nuclear AR levels in prostate cancer cells. LNCaP cells and GFP-AR-expressing PC3 cells were incubated with: *A* and *D*, vehicle alone; *B* and *E*, IL-6 (10 ng/ml); or *C* and *F*, DHT (1 nM). After 24 h, they were subjected to immunofluorescent staining with an anti-AR antibody or confocal microscopy. *Arrows*, nuclear staining. *A*, ×400; *B*, ×630; *C*, ×400. *Bar*, 15 μm. *G*, LNCaP cells were treated with medium alone, IL-6 (10 ng/ml), or DHT (1 nM) for 24 h; then nuclear proteins were collected and subjected to Western blot analysis (20 μg/lane) and probed with anti-AR antibody. Bands were measured by densitometry. The blot shown here is the result of a typical experiment. Data are reported as percentage of AR protein relative to untreated cells, which was set at 100%.

observation is consistent with several other nonandrogenic compounds that have been reported to stimulate AR. For example, several substances that bind to membrane receptors up-regulate the activity of AR (30–34). Furthermore, that IL-6 increased nuclear AR in the absence of androgen suggests that IL-6 may mediate its effect in part through unmasking the nuclear localization sequence. However, our observation that IL-6 did not induce androgen-responsive genes in the DU145 cells transfected with the AR is in apparent conflict with these findings. Taken together, these observations suggest that the AR is required but not sufficient for IL-6 to mediate androgen-like activity. It is possible that DU145 cells are deficient in some cofactor required for IL-6 to mediate its androgen-like activity.

Several signal transduction molecules are potential mediators of the ability of IL-6 to activate AR. For example, the observations that several MAPK inhibitors blocked IL-6-mediated androgen-responsive promoter activation suggest that IL-6 induces AR activity through a MAPK-dependent pathway. These data are consistent with the ability of IL-6 to induce the Ras signal transduction pathways (43), which depends on Raf, MEK, and MAPK. These data are also consistent with the previous report that IL-6 requires the growth factor receptors ErbB2 and -3 to mediate signaling in LNCaP cells (20). However, the observation that the PI-3-kinase inhibitor, wortmannin, had no effect on IL-6-mediated PSA promoter induction suggests that this pathway is not important for androgen responsiveness, although IL-6 activates the PI-3-kinase pathway in several cell types including LNCaP cells (25, 26, 35, 36). Finally, the recent report that the inhibition of IL-6-mediated activation of STAT3 diminishes the ability of IL-6 to induce androgen-like activity suggests that the Jak/STAT pathway is an important component of IL-6-mediated activation of the AR

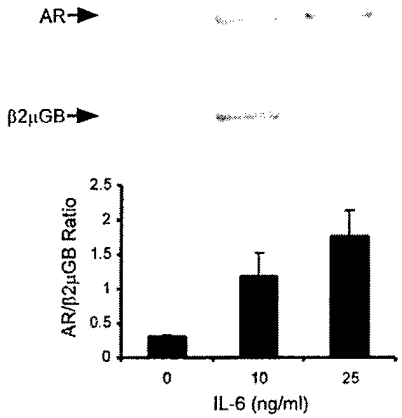


Fig. 7 IL-6 induces AR mRNA levels in LNCaP cells. LNCaP cells were treated with IL-6 (0, 10, and 25 ng/ml) for 24 h. Total RNA was collected and subjected to semiquantitative RT-PCR using primers for AR (*AR*) and β2-microglobulin (*β2μGB*). PCR products were measured by densitometry. Data are reported as the mean (± SE) ratio of AR: β2μGB mRNA from three independent experiments. The gel shown is the result of a typical experiment.

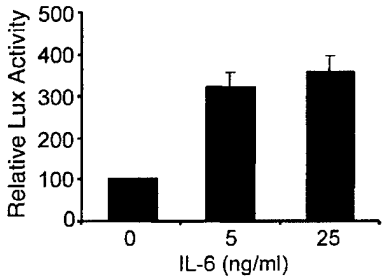


Fig. 8 IL-6 stimulates AR gene promoter activity in LNCaP cells *in vitro*. LNCaP cells were cotransfected with pRL-CMV (for normalization using the dual lux assay) and the reporter plasmid, p2.3kAR-Lux. Cells were then treated with IL-6 (0, 5, 25 ng/ml) for 24 h. After incubation, lux levels were measured using a luminometer. Data are reported as the mean (±SE) lux activity relative to the untreated cells, which was set as 100%, from three independent experiments.

(19). These previous reports, taken together with our current data, suggest that both MAPK and Jak/STAT signal transduction pathways are required to mediate the effect of IL-6 on AR function.

In addition to increased AR function, the finding that IL-6 increased AR expression at both mRNA and protein levels suggests that IL-6 enhances androgen activity by up-regulating the AR levels. This is consistent with the heterogeneous expression of AR that occurs as prostate cancer progresses (44–46). Furthermore, it has pathophysiological implications because there are low levels of androgens even after androgen ablation therapy (47). Specifically, if IL-6 increases AR levels in prostate cancer cells, then it follows that these cells may have increased sensitivity to the low levels of androgens in the androgen-ablated patient, enabling these cells to respond to androgens. In effect, what appears to be an androgen-independent tumor, based on its progression in an androgen-ablated patient, would

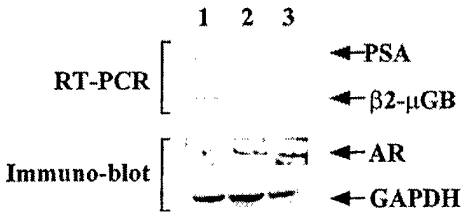


Fig. 9 Increased AR levels in LNCaP cells cause reducing PSA expression. LNCaP cells were transfected with empty vector (*Lane 1*), 0.4 μg or 2 μg of AR plasmid (*Lanes 2* and *3*). Total RNA was collected and subjected to semiquantitative RT-PCR using primers for PSA and β2-microglobulin (β2μGB). PCR products were resolved by electrophoresis. Protein from the same cell lysate 40 μg were resolved by SDS-PAGE gel electrophoresis followed by AR and GAPDH immunoblotting.

really be hypersensitive to androgens. This hypothesis is consistent with the fact that the LNCaP cells, which are androgen responsive, were originally isolated from a patient in whom androgen ablation failed (16, 17).

The clinical observations that elevated IL-6 levels are frequently associated with androgen-independent prostate cancer have predicted an important role for IL-6 signaling in prostate cancer androgen-independent progression (40, 48–51). For example, serum IL-6 levels were shown to predict survival in prostate cancer patients (49). IL-6 levels in the serum of these patients range between 10 and 700 pg/ml, with occasional findings of >1 ng/ml (40, 48–51). In our study, we used nanogram levels of IL-6, which may reflect the IL-6 levels found in the tumor microenvironment. This postulation is supported by the observations that several prostate cancer cell lines produce nanogram levels of IL-6[4] (5, 52). However, because of the wide range of IL-6 levels in men afflicted with prostate cancer, the *in vitro* application of IL-6 at the nanogram level requires cautious interpretation in terms of its bearing on *in vivo* pathophysiology. Moreover, from our data, which showed that IL-6 very likely contributes to the growth of advanced-prostate-cancer growth and that BIC abolishes the effect of IL-6, one might expect that this antiandrogen will be a powerful therapeutic agent in treating the advanced-prostate-cancer patient. Unfortunately, trials of BIC monotherapy have not demonstrated efficacy in the clinical setting (reviewed in Ref. 53). Because of the fact that the cancer tissue is heterogeneous, it is likely that there is a subpopulation of cancer cells that can survive via alternative mechanisms to which IL-6 may contribute independent of the AR signaling pathway.

The demonstration of IL-6-induced activation of the AR promoter and the subsequent increase of AR mRNA and protein is the first report of non-androgen-mediated induction of the human AR promoter. Other than IL-6, only androgens have been reported to activate the human AR promoter (54–56). Grad *et al.* (57) demonstrated the regulatory motif within the AR gene-coding region required for AR auto-up-regulation. Whether or not IL-6 uses this same motif is currently unknown.

[4] Unpublished observations.

Furthermore, the *trans*-acting factors and *cis*-acting promoter sites through which IL-6 mediates induction of the AR promoter are currently unknown. The induction of AR mRNA and protein expression described thus far has been rare (58–60). Thus, our data provide a new mechanism through which AR levels are controlled.

Advanced CaP is associated with increased serum IL-6 levels (50). The source of IL-6 in the CaP patients is not known. However, we have previously demonstrated that androgen down-regulates IL-6 expression in LNCaP cells (61). Furthermore, we and others have shown that orchiectomy induces IL-6 expression in mice (62, 63). Thus, it follows that androgen deprivation may account for the increased serum IL-6 levels observed in patients with advance prostate cancer. These observations, taken together with results from the present study, suggest that androgen deprivation may induce IL-6 expression in prostate cells, which, in turn, will induce androgen-like activity in the CaP cells. This could lead to androgen-independent tumor growth. Intriguingly, it is possible that, as IL-6 induces androgen-like activity, this will in turn create a negative feedback on IL-6 production.

In summary, our study provides evidence that IL-6 increases androgen-like action by up-regulating the AR expression in CaP cells. Furthermore, our data suggest that IL-6 activates the AR in the absence of androgen. Together, these mechanisms can account for the contribution of IL-6 to the progression of prostate cancer. Furthermore, these results suggest that androgen-deprivation therapy may promote the progression of CaP to an androgen-independent state through increasing expression of IL-6.

Acknowledgments

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The Prostate 48:47–53 (2001)

Anti-Interleukin-6 Monoclonal Antibody Induces Regression of Human Prostate Cancer Xenografts in Nude Mice

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BACKGROUND. Despite clinical associations and in vitro data suggesting that autocrine interleukin-6 (IL-6) production contributes to prostate cancer progression or chemotherapy resistance, there have been no reports that explore the role of IL-6 on prostate tumors in vivo. In the present study, we investigated the effect of IL-6 inhibition on the growth of human prostate cancer xenografts in nude mice.

METHODS. To determine if autocrine IL-6 production contributes to prostate cancer growth and chemotherapy resistance in vivo, xenografts of a human prostate cancer cell line that produces IL-6 (PC-3) were established in nude mice. The mice were randomly divided into four treatment groups: (1) saline (vehicle control) + murine IgG (isotype control); (2) etoposide + murine IgG; (3) saline + anti-IL-6 monoclonal antibody; and (4) etoposide + anti-IL-6 monoclonal antibody. Tumors were measured twice weekly during a 4-week treatment period. At the conclusion of the study, all mice were sacrificed, and in addition to final volume, tumors were evaluated for the degree of apoptosis by TUNEL analysis.

RESULTS. Anti-IL-6 Ab (with saline or etoposide) induced tumor apoptosis and regression (~60% compared to initial tumor size). Etoposide alone did not induce tumor regression or apoptosis in this animal model, and there was no synergy between anti-IL-6 Ab and etoposide.

CONCLUSIONS. These studies suggest that IL-6 contributes to prostate cancer growth in vivo, and that targeting IL-6 may contribute to prostate cancer therapy. *Prostate 48:47–53, 2001*

KEY WORDS: **cytokine; interleukin-6; antibody; prostate cancer; chemotherapy**

INTRODUCTION

Prostate cancer is the most common cancer diagnosed in men and the second leading cause of cancer death among men in the United States. In 1999, it was estimated that 179,300 patients were diagnosed with prostate cancer, and 37,000 patients died from the disease [1]. Radical prostatectomy can be curative in patients with localized prostate cancer. Unfortunately, many patients have an advanced form of the disease at the time of diagnosis, and require systemic androgen-ablation therapy. Initially the cancer appears as an androgen-sensitive phenotype and is responsive to this treatment. However, after a median time of 12–18 months, it commonly recurs as a hormone-refractory phenotype that is also resistant to other therapeutic

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modalities including chemotherapy [2]. The precise mechanism of drug resistance in prostate cancer is not fully understood, but the secretion of protective factors by these tumors may play a role.

The cytokine interleukin-6 (IL-6) has been implicated in a number of pathophysiologic processes including stimulation of tumor proliferation [3]. In the past few years, evidence has been accumulating that IL-6 may contribute to the progression of prostate cancer [4]. For example, IL-6 serum levels are correlated with morbidity and tumor burden of prostate cancer patients [5]. Furthermore, the addition of anti-IL-6 antibody to the growth medium of androgen-independent prostate cancer cell lines has been shown to inhibit cell growth [6,7]. Additionally, inhibition of IL-6 activity enhances the cytotoxic activity of certain chemotherapeutic agents in prostate cancer cell lines that are resistant to the drugs [8]. In spite of the many studies demonstrating that IL-6 promotes prostate cancer proliferation and survival in cell culture, there has been no in vivo evidence to confirm that IL-6 contributes to prostate cancer growth. Accordingly, to determine if IL-6 contributes to prostate cancer progression, we examined the effect of inhibiting IL-6 activity on prostate cancer progression in mice implanted with a human prostate cancer xenograft.

MATERIALS AND METHODS

Cell Lines

The hormone-independent prostatic carcinoma cell lines PC-3 and DU145, and the hormone-dependent cell line, LNCaP (ATCC) were cultured in complete medium (RPMI 1640 with L-glutamine supplemented with 10% heat-inactivated fetal bovine serum, and 1% penicillin/streptomycin solution containing 10,000 units/ml penicillin G and 10,000 μg/ml streptomycin). All three cell lines were grown in a humidified incubator at 37°C in 5% CO_2. Cells were treated with trypsin-EDTA, washed, and resuspended in complete medium prior to their use in cytotoxicity assays.

Antibodies

Anti-hIL-6 (CLB, Amsterdam, The Netherlands) antibody is a mouse monoclonal (subtype IgG1) specific for human IL-6 [9]. Mouse $IgG1_k$ (Sigma, St. Louis, MO) was used as an isotype control antibody.

Etopsoide

Etoposide (Sigma) was dissolved in 1 ml DMSO to a final stock concentration of 25 mg/ml and stored at 4°C. The stock solution was diluted to the indicated concentrations in complete medium (in vitro experiments) or normal saline (in vivo experiments) immediately prior to use.

IL-6 Measurements in Cell Culture

Cells were grown in 10-cm polystyrene tissue culture dishes. Confluent cells were washed once with phosphate-buffered saline and then incubated for 48-hr in complete medium, at which time the supernatant was collected and stored at −20°C until assayed. IL-6 concentration was measured using a commercial ELISA kit (Quantikine Human IL-6 ELISA Kit; R&D, Systems Inc., Minneapolis, MN) as directed by the manufacturer.

In some cases, IL-6 levels were measured following the addition of anti-IL6 antibody. In these instances, cells were plated in 6-well polystyrene tissue culture plates at a density of 5×10^5/well in 2.5-ml complete medium. Anti-IL6 monoclonal antibody, isotype control (final concentration of 500 ng/ml), or complete medium was added to appropriate wells and cells were incubated at 37°C. Supernatant samples were collected from each well at 24, 48, and 72 hr and stored at −20°C until ELISA was performed.

Serum IL-6 Levels

At the time of sacrifice, blood samples were collected via cardiac puncture and centrifuged at 2,700 rpm for 10 min. The serum was removed from each sample and stored at −80°C until assayed for IL-6 using the B9 cell IL-6 bioassay as previously described [10].

In Vitro Cytotoxicity Experiments

Cell lines were seeded in 96-well plates at a density of 2×10^3 cell/well in 100-μl complete medium. Either anti-IL6 or isotype control antibody was added at a concentration of 2 μg/ml (final concentration to be 500 ng/ml) in 50 μl of complete medium, and etoposide was added in a 50-μl/volume to reach a final concentration of 0.1 or 10 μg/ml (approximate ID_{25} and ID_{50}, respectively; data not shown). Saline vehicle was added to cells that did not receive etoposide. The cells were then incubated for 48 hr. Cell viability was then determined using an MTS assay (Promega, Madison, WI) as directed by the manufacturer. Cytotoxicity was calculated as follows:

$$\%\,\text{cytotoxicity} = [1 - (\text{absorbance of experimental wells}/\text{absorbance of control wells})] \times 100.$$

Mice

Eight-week-old nude (nu/nu) mice (Charles River Laboratories, Wilmington, MA) were kept in a specific pathogen free colony, in microisolator cages, and were fed sterile rodent chow and sterile water ad libitum. All protocols were approved by the University of Michigan Animal Care and Use Committee.

In Vivo Experiments

Confluent PC-3 cells were harvested by trypsinzation, washed twice with PBS and resuspended at a density of 1×10^7 cells/ml. The mice were injected subcutaneously with 100 μl of the tumor cell suspension (10^6 cells) combined with 100 μl of Matrige® (Bectin-Dickson, Bedford, MA). The mice were monitored for tumor growth, and when tumors were detected by palpation, measurement of the tumors began. Tumor volumes were calculated by the formula: Volume = [(minimum measurement)^2 (maximum measurement)] ÷ 2 [as described in Ref. [11]]. Tumors were measured every other day, and when tumors reached a volume of 126 mm^3 the mice were randomly assigned to one of four treatment groups (n = 10/group). Treatment groups included isotype + saline, isotype + etoposide, anti-IL-6 + saline, and anti-IL-6 + etoposide. The treatment regimen consisted of weekly i.p. injection of anti-IL6 or isotype antibody at 500 μg/mouse/week as previously described [12] and daily i.p. injections of etoposide at 50 mg/m^2/day, which is the human-equivalent dose [13], or an equal volume of saline. Mouse IL-6 does not react with human IL-6 receptor [14]. Thus, using an anti-human-IL-6 alone will inhibit the IL-6 specifically produced by the human prostate cancer cells. Treatment continued for 4 weeks, during which time the tumors were measured on a twice-weekly basis. At the conclusion of the study all mice were sacrificed, and blood and tissue samples were collected for further analysis.

Tumor Histopathology and Detection of Apoptosis

Excised tumors were placed in 10% formalin, embedded in paraffin and sectioned at 10 μM thickness. Sections were examined utilizing standard hematoxylin and eosin (H&E) staining for routine histopathology. To evaluate apoptosis, sections were deparaffinized, rehydrated, and subjected to terminal deoxytransferase UTP end-labeling (TUNEL) analysis using ApopTag® Plus Peroxidase Kit (Intergen, Purchase, NY) according to the manufacturer's directions. The number of apoptotic nuclei per 200X field (averaged from three random 200X fields) was determined for each section by an investigator that was blinded to the samples as previously described [15].

Statistical Analysis

To determine differences among treatment groups for tumor size, two-way analysis of variance (ANOVA) was used followed by Fisher's least significant difference for post-hoc analysis. Statistical significance was determined at $P < 0.05$.

RESULTS

IL-6 Secretion by Prostate Cancer Cell Lines

The detection of IL-6 secretion by prostate cancer cell lines is fairly inconsistent between laboratories [6,7,16]. Thus, it was critical to determine the IL-6 expression of various cell lines in our laboratory prior to proceeding with an in vivo challenge. Accordingly, we measured the amount of IL-6 secreted into the culture supernatant of various prostate cancer cell lines. PC-3 cells secreted the most IL-6 followed by DU-145 cells (Fig. 1A). IL-6 was undetectable in LNCaP cell culture supernatant (Fig. 1A).

Effect of IL-6 Antibody Treatment on IL-6 Levels in PC-3 Cell Culture Supernatant

In order to provide a maximum challenge to our ability to inhibit IL-6, we performed the remaining experiments with the PC-3 cells, which secreted the highest levels of IL-6. To confirm that the anti-IL-6 antibody we were using effectively inhibited IL-6 levels over a length of time, we incubated PC-3 cells with 500 ng/ml of either anti-IL-6 or isotype control antibody for 24, 48, and 72 hr, then measured IL-6 levels using ELISA. Anti-IL-6 antibody decreased the detection of IL-6 by ≥ 50% at all three time points compared to the isotype antibody (Fig. 1B).

Effect of Anti-IL-6 and Etoposide on Cell Proliferation of PC-3 Cells

To determine if anti-IL-6 antibody enhances the etoposide-mediated cytotoxicity of prostate cancer cells, we incubated prostate cancer cells with antibody and etoposide, then measured viable cell number. Anti-IL-6 antibody alone decreased the number of viable cells by approximately 10% (Fig. 2). Etoposide alone at low (0.1 μM) and high (10 μM) doses induced approximately 5% cytotoxicity. Anti-IL-6 antibody combined with the high dose of etoposide induced approximately 25% cytotoxicity, thus demonstrating a synergistic effect between etoposide and anti-IL-6 antibody in vivo. These in vitro data provided the rationale to pursue the ability of IL-6 antibody to modulate prostate cancer cell growth in vivo.

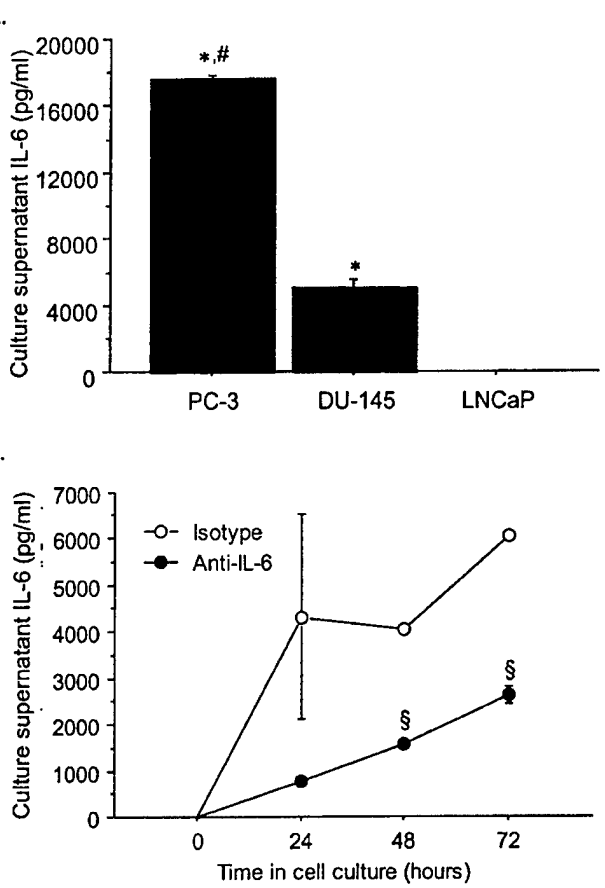

Fig. 1. Anti IL-6 antibody diminished ELISA detectable IL-6 from PC-3 cells. **A:** The indicated prostate cancer cell lines were plated at a density of 5×10^6 cells/10 ml in 10 cm tissue culture plates and cultured for 48 hr. Supernatant was then collected and subjected to ELISA for IL-6. **B:** PC-3 cells were plated in 6-well polystyrene tissue culture plates at a density of 5×10^5/well in 2.5 ml complete medium. Anti-IL-6 monoclonal antibody or isotype control (final concentration of 500 ng/ml) was added and cells were incubated at 37°C. Supernatant samples were collected from each well at 24, 48, and 72 hr and subjected to ELISA for IL-6. All time points were run on the same plate. Both assays were performed in triplicate. $^*P < 0.01$ vs. LNCaP, $^{\#}P < 0.01$ vs. DU-145, $^{\S}P < 0.05$ vs. isotype.

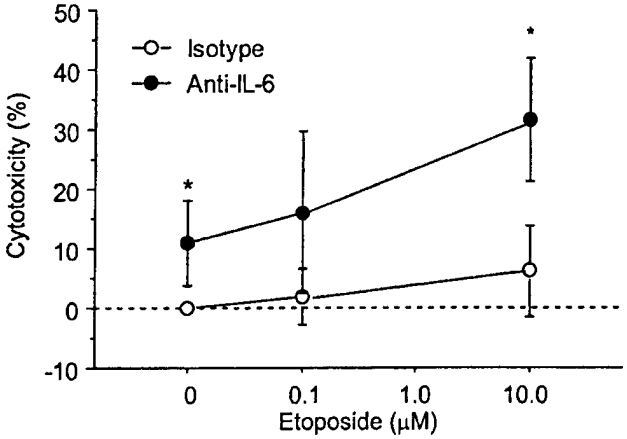

Fig. 2. Inhibition of IL-6 induces cytotoxicity of PC-3 cells in vitro. Cell lines were seeded in 96-well plates at a density of 2×10^3 cells/well in 100 μl complete medium. Either anti-IL6 or isotype control antibody was added at a concentration of 2 μg/ml (final concentration to be 500 ng/ml) in 50 μl of complete medium. Etoposide was added in a 50 μl/volume to reach a final concentration of 0.1 μg/ml or 10 μg/ml (approximate ID_{25} and ID_{50}, respectively; data not shown). Saline vehicle was added to wells not receiving etoposide. The cells were then incubated for 48 hr. Cell viability was then determined using an MTS assay and cytotoxicity was determined as described in the Methods section. The assay was performed in triplicate. $^*P < 0.05$ vs. isotype.

Tumor Response

Based on our observation that inhibition of IL-6 alone inhibited PC-3 survival in addition to enhancing-mediated cytotoxicity in vivo, we next explored if inhibiting IL-6 would mimic these effects in vivo on established prostate cancer tumors. To accomplish this, PC-3 cells were subcutaneously injected into nude mice. The tumors were allowed to develop until they were approximately 126 mm^3, at which time anti-IL-6 or isotype antibody and etoposide administration was initiated. Antibody was administered at a level that inhibited IL-6 bioactivity by approximately 20% (based on B9 bioassay; data not shown). Treatment was continued for a 4-week period. Using an ELISA specific for human IL-6 (and non-cross-reactive with murine IL-6), we determined that serum human IL-6 levels were 30.1 ± 10.4 pg/ml in tumor-implanted saline + isotype control mice, compared to undetectable levels in mice not implanted with tumor, demonstrating that the tumors produced IL-6 in vivo. The tumors in the isotype-treated mice had a continuous, albeit slow, tumor growth rate, whereas the tumors in the isotype + etoposide-treated mice did not grow (Fig. 3). In contrast, the final tumor volumes were reduced by approximately 60% compared to their initial size in the mice receiving anti-IL-6 or anti-IL6 + etoposide. Furthermore, they were approximately 75% smaller than the tumors in the mice receiving isotype alone (Fig. 3). There was no significant difference between the tumor volumes in the mice treated with isotype alone compared to the mice treated with etoposide alone.

Apoptosis

We evaluated the effect of anti-IL-6 antibody and etoposide on the amount of apoptosis present in the PC-3 tumors. Routine histological evaluation of the tumors did not demonstrate any differences among the treatment groups. However, administration of anti-IL-6 antibody was associated with marked apop-

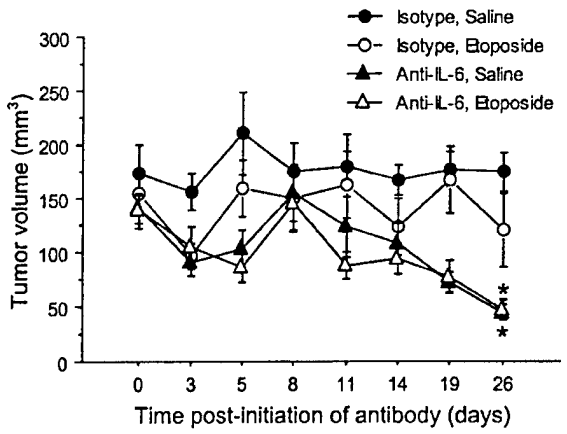

Fig. 3. Inhibition of IL-6 induces PC-3 xenograft regression in mice. PC-3 cells (10^6) in a Matrigel slurry were subcutaneously implanted in male nu/nu mice. When tumors reached 126 cm^3, weekly i.p. injections of anti-IL6 or isotype antibody (500 μg/mouse) and daily i.p. injections of etoposide (50 mg/m^2) or saline vehicle were initiated. Tumors were measured twice-weekly for 4 weeks. There were 10 animals/group. Results are shown as mean ± SD. $^*P < 0.01$.

tosis in the tumors from both the saline vehicle and etoposide treated mice, compared to the moderate level of apoptosis in the tumors from the isotype and isotype + etoposide-treated mice (Fig. 4). These data demonstrate that IL-6 has an anti-apoptotic action in PC-3 cells in vivo.

DISCUSSION

A large body of evidence has accumulated that suggests IL-6 contributes to prostate cancer progression. The evidence includes both clinical observations that increased levels of IL-6 are associated with increasing grade of prostate cancer in patients [5,17,18] and in vitro experiments that have demonstrated that IL-6 promotes prostate cancer cell growth and prevents chemotherapeutic-mediated cytotoxicity [7,19]. In the current study, we provide the first demonstration that IL-6 activity promotes prostate cancer growth in vivo.

Our data demonstrate that high levels of IL-6 are secreted by PC-3 and DU-145 cells, whereas IL-6 levels were not detectable using ELISA methodology in LNCaP cells. These findings are consistent with previous reports on IL-6 secretion by prostate cancer cell lines [6,8]. PC-3 and DU-145 cells are androgen non-responsive, whereas LNCaP cells are androgen-responsive. Thus, these results suggest that loss of androgen responsiveness is associated with increased IL-6 expression. This postulation is consistent with the observations that elevation of serum IL-6 levels is associated with increasing grade of prostate cancer [5,18,20]. A cause and effect cannot be determined based on the current data. However, it has been previously reported that the androgen dihydrotestosterone inhibits IL-6 expression in prostate cancer cells [16] and that orchiectomy increased IL-6 expression in murine bone marrow [10]. Thus, it is plausible that loss of androgen-response promotes IL-6 expression.

Prostate cancer is poorly responsive to chemotherapy. Therefore, a mechanism to enhance chemotherapeutic killing of tumors would be a boon for prostate cancer patients. Borsellino et al. [7] have reported that inhibition of IL-6 activity enhances chemotherapeutic killing of prostate cancer cell in vitro. However, this effect has not been reported in vivo. In the current study, the cell type and therapeutic agent were chosen based on in vitro studies, and the human equivalent dose of etoposide was used to calculate the dosage administered to the mice. However, we did not observe an effect of etoposide on the PC-3 in vivo. In contrast, tumors responded to anti-IL-6 antibody, although the combination of anti-IL-6 and etoposide did not significantly enhance this inhibitory effect compared to anti-6 alone. Taken together, these data suggest that inhibition of IL-6 does not enhance etoposide-mediated killing in vivo. However, they clearly demonstrate that inhibition of IL-6 alone, or in the presence of etoposide, induces regression of PC-3 tumors. This observation provides in vivo evidence that IL-6 contributes to prostate cancer cell growth, and that inhibition of its activity promotes tumor regression.

This ability to inhibit IL-6 activity in humans has been previously demonstrated in several clinical trials using murine monoclonal antibodies in patients with multiple myeloma [21,22]. Early trials demonstrated the feasibility of blocking IL-6 activity in this manner, and that such therapy had beneficial effects [21]. These trials also revealed certain limitations to anti-IL-6 therapy using murine monoclonal antibodies. One such limitation is that in some patients with advanced disease, IL-6 levels were so high that the antibody was unable to neutralize them [22]. Another limitation is that development of antibodies to mouse immunoglobulin may result in rapid clearance of the murine monoclonal antibody and diminished efficacy of treatment [23]. This problem has been addressed through the chimerization [23,24] and humanization [24] of murine anti-IL-6 antibodies. Tsunenari et al. [24] demonstrated reduced antigenicity of chimeric antibodies and even lower antigenicity of humanized murine antibodies (24) while a later study by van Zaanen et al. [23] showed no induction of human anti-chimeric antibodies in multiple myeloma patients receiving chimeric anti-IL-6 antibodies. Overall, these studies suggest that inhibition of IL-6 activity in prostate cancer patients is achievable.

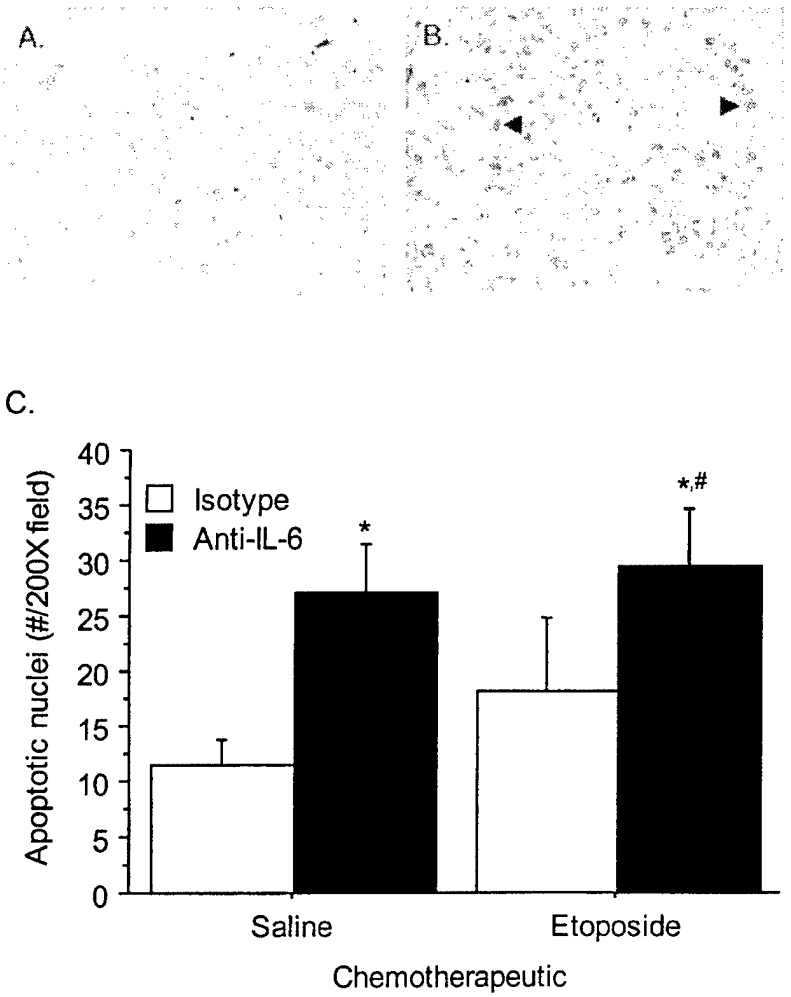

Fig. 4. Inhibition of IL-6 induces apoptosis in PC-3 tumors in mice. Tumors were excised from mice 4 weeks after initiation of anti-IL6 and etoposide as described in Fig. 3. Breaks in DNA were determined by labeling 3′OH termini using terminal deoxytransferase and staining with peroxidase. Tumor sections are shown from (**A**) isotype-treated mice and (**B**) anti-IL-6 treated mice. Apoptotic nuclei are dark brown (arrowheads). Original magnification 100 ×. **C**: To determine the degree of apoptosis, the number of apoptotic nuclei/200 × field (average of triplicate) were determined by an investigator blinded to the samples. *$P < 0.01$ compared to the saline + isotype mice; *$P < 0.05$ compared to the etoposide + isotype mice. Data are shown as mean ± SD of 4 mice/group.

The mechanism through which IL-6 contributes to overall prostate tumor growth is not clear. There are conflicting reports regarding the effect of IL-6 on prostate cancer cell proliferation in vitro [4]. Thus, it is unclear if IL-6 directly contribute to tumor growth through stimulation of cell proliferation. In addition to increased cell proliferation, a tumor may enlarge due to decreased apoptotic death of cell. The observation that IL-6 has been demonstrated to have anti-apoptotic action in several cell types [19,25,26] including the prostate cancer cell lines LNCaP and PC-3 (27) led us to evaluate IL-6's effect on apoptosis in the prostate cancer xenografts. Our observation that the level of apoptosis in tumors of mice that received anti-IL-6 compared to those who received isotype control antibody demonstrates that IL-6 protects prostate cancer cells from apoptosis in vivo. These findings are consistent with the in vitro results of Chung et al. [27] who demonstrated the antiapoptotic effects of IL-6 in PC-3 and LNCaP cell lines, and showed that this effect is the result of IL-6 activation of phosphatidylinositol (PI)-3 kinase. These previous reports, taken together with the currently reported murine studies, suggest that inhibition of apoptosis is one mechanism through which IL-6 contributes to prostate cancer progression.

CONCLUSION

In summary, the current study demonstrates that anti-IL-6 antibody induces apoptosis and regression of established PC-3 tumors in mice. However, the in vivo

data do not support the in vitro observations that IL-6 enhances etoposide-mediated killing. These data, combined with the clinical reports that IL-6 is associated with prostate cancer stage [17,18,28], provide compelling evidence that IL-6 contributes to prostate cancer progression and suggests that targeting IL-6 may induce prostate cancer regression.

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Mini Review

Interleukin-6 and prostate cancer progression

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Abstract

Prostate cancer, while initially dependent on androgens for proliferation, progresses to an androgen-independent state. Evidence has been accumulating that interleukin-6 (IL-6) may contribute to prostate cancer progression. Serum levels of IL-6 correlate with prostate tumor burden and patient morbidity. The prostate tissue itself appears to be a source of IL-6 and its receptor. Furthermore, experimental data suggest that IL-6 is an autocrine and paracrine growth factor for androgen-independent prostate cancer cell lines. For example, inhibition of IL-6, with anti-IL-6 antibody, sensitizes androgen-independent prostate cancer cells to chemotherapeutic agents in vitro. Finally, IL-6 activates a variety of signal transduction cascades, some which stimulate androgen receptor activity, in prostate cancer cells. These data suggest that targeting IL-6 may have multiple benefits in prostate cancer patients.

Keywords: Cytokine; Androgen receptor; Signal transduction; Chemotherapy; Androgen independence

1. Introduction

Prostate cancer is the most common cancer diagnosed in men and the second leading cause of cancer death among men in the US. In 1999, it was estimated that 179 300 patients were diagnosed with prostate cancer, and 37 000 patients died from the disease [1]. The increased incidence of prostate cancer in this country is unparalleled by any other tumor in the last 20 yr, and associated mortality has steadily increased [2].

Prostate cancer initially occurs as an androgen-dependent tumor. Thus, androgen-deprivation is a commonly used therapeutic strategy for prostate cancer. While the initial response rate is excellent, the cancer eventually recurs in the androgen-deplete state. The tumor, now termed androgen independent, typically progresses resulting in death of the patient. The mechanisms through which androgen independence develops are unknown. However, putative mechanisms can be divided into those that are dependent on activation of the androgen receptor (AR), either through ligand-dependent (i.e. androgen) or ligand-independent methods [3], or those mechanisms that activate prostate cancer proliferation through non-AR-dependent growth factors.

Among the many putative prostate cancer growth factors is the cytokine, interleukin-6 (IL-6). IL-6 has many physiologic roles and has been implicated in a number of pathophysiologic processes. A variety of tumor types are stimulated by IL-6, including melanoma, renal cell carcinoma, Kaposi's sarcoma, ovarian carcinoma, lymphoma and leukemia, multiple myeloma, and prostate carcinoma [4]. In the last few years, evidence has been accumulating that IL-6 may contribute to the progression of prostate cancer. The purpose of this article is to review the biology of IL-6 and its receptor, and to summarize information from the literature concerning their association with prostate cancer and their potential role in its pathophysiology.

2. Biology of IL-6 and its receptor

2.1. Interleukin-6

IL-6 is a 21–28 Kd protein containing 184 amino acids following cleavage of a 28 aa signal peptide [5].

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IL-6 belongs to the "IL-6 type cytokine" family that also includes leukemia inhibitory factor, IL-11, ciliary neurotrophic factor, cardiotrophin-1 and oncostatin M [6]. In the normal homeostatic state, IL-6 levels are typically very low. However, in response to the appropriate stimulus (e.g. inflammation), a wide variety of cells produce IL-6. Many physiologic functions are attributed to IL-6 including promotion of antibody production from B lymphocytes, modulation of hepatic acute phase reactant synthesis, promotion of osteoclastic-mediated bone resorption, and induction of thrombopoiesis [7].

2.2. IL-6 receptor and signal transduction

IL-6 mediates its activity through the IL-6 receptor complex, which is composed of two components; an 80 Kd transmembrane receptor (IL-6Rp80, IL-6R, α-subunit) that specifically binds IL-6, but has no signaling capability and a 130 Kd membrane glycoprotein (gp130) that mediates signal transduction following IL-6R binding [8]. Other members of the IL-6 family of cytokines have specific α-subunits, but share gp130 as a component critical for signal transduction [9]. The human IL-6R consists of 468 aa, including a signal peptide of 19 aa, an extracellular region of 339 aa, and a membrane-spanning region of 28 aa. Due to *N*-glycosylation, the molecular weight of IL-6R is 80 kDa instead of 50 kDa as predicted from its aa sequence [10]. Gp130 consists of 918 aa, including a leader sequence of 22 aa, an extracellular region of 597 aa, a membrane-spanning region of 22 aa, and a cytoplasmic region of 277 aa [1]. IL-6 binds to the IL-6R with low affinity [12,13] and induces disulfide-linked homodimerization of gp130 [13]. Gp130 has no intrinsic IL-6 binding capacity, but its complex formation with the IL-6R promotes high affinity binding of IL-6 as well as signal transduction [11]. The resulting compound is a hexameric complex consisting of two molecules each of IL-6, IL-6Rp80, and gp130 [12]. The homodimerization of gp130 activates associated tyrosine kinases (TYK) that subsequently cause gp130 phosphorylation, thus initiating signal transduction [13]. Mutation analysis of the cytoplasmic region of gp130 has led to identification of multiple regions implicated in signal transduction [14]. This reflects the pleiotropic nature of IL-6, in that separate sets of signals appear to be required to regulate such a diverse array of genes and cellular responses. Multiple sites are phosphorylated on tyrosine residues of gp130; however, gp130 does not appear to have any inherent tyrosine kinase activity. Instead, gp130 associates with tyrosine kinases such as Janus kinase family members JAK/TYK [15] and possibly others such as the src-related tyrosine kinase HcK [16]. Tyrosine residues within receptors mediate signal transduction through the recruitment of either src homology 2 (SH2) domains or phosphotyrosine binding domains. Gp130 has six tyrosine residues in its cytoplasmic domain [17]. By using chimeric receptors, consisting of the extracellular domain of growth hormone receptor and the transmembrane and cytoplasmic domain of gp130 with progressive C-terminal truncations, Yamanaka et al. demonstrated that the tyrosine residue with the YXXQ motif of the membrane-proximal of gp130 could generate the signals for growth arrest, macrophage differentiation, down-regulation of c-myc and c-myb, induction of junB and IRF1 and STAT3 activation [18]. This observation was further confirmed by Tomida et al. who constructed chimeric receptors by linking the transmembrane and intracellular regions of mouse gp130 to the extracellular domains of the human granulocyte macrophage colony-stimulating factor receptor alpha and beta chains. Using the full-length cytoplasmic domain and mutants with progressive C-terminal truncations or point mutations, they showed that the two membrane-distal tyrosines with the YXXQ motif of gp130 were critical not only for STAT3 activation, but also for growth arrest and differentiation of cells [19]. Thus, certain tyrosine residues play pivotal roles in gp130 mediated signal transduction regulating cell growth, differentiation, and survival.

In addition to the transmembrane IL-6R, a soluble form of IL-6R (sIL-6R) exists. There is evidence that the soluble form is produced by either proteolytic cleavage of the 80 kDa subunit [20,21] or differential splicing of mRNA [22]. Several recent reports that describe a truncated form of IL-6 receptor mRNA identified through RT-PCR from a human hepatoma cell line (HepG3), mononuclear cells from inflammatory bowel disease patients, acute myeloblastic leukemia (AML) patient cell lines, and primary AML blast cells [23–25] support alternative splicing as a mechanism of sIL-6R production. Although the sIL-6R does not posses a transmembrane component, it can still bind to IL-6 and the ligand bound sIL-6R. IL-6 complex activates signal transduction and biological responses through membrane-bound gp130 [26]. Thus, IL-6 can still affect cells that do not express transmembrane IL-6R, as long as they express gp130.

A few signaling pathways for IL-6 have been described that may act simultaneously in certain types of cells or preferentially in others. Some of the IL-6 response genes are targets of the tyrosine phosphorylation pathway involving Janus kinases (JAK) and the signal transducer and activator of transcription (STAT) family of nuclear factors. There appears to be constitutive association between gp130 and JAK1, JAK2, and TYK2. Tyrosine kinase activity is activated secondary to gp130 dimerization induced by the binding of IL-6 to IL-6R, probably through conformational change of the receptor complex [27]. Following the phosphorylation of gp130, STAT family members associate with gp130

dimers and act as substrates for JAK/TYK. In the case of STAT3, tyrosine phosphorylation occurs at a single residue (Tyr705) that is located in a conserved SH2 domain [28]. Upon tyrosine phosphorylation, STATs (including STAT1, STAT3, and STAT5) translocate to the nucleolus as homodimers or heterodimers and bind to specific consensus DNA sequences of target-gene promoters and activate transcription [14,29–35]. In addition to tyrosine phosphorylation, STAT3 is phosphorylated at a single serine residue (Ser727) in response to IL-6 [36]. A recent report by Schuringa et al. suggests that, independent of ERK-1 or JNK-1, IL-6-induced STAT3 transactivation and phosphorylation involves a gp130-signaling cascade that includes Vav, Rac-1, MEKK and SEK-1/MKK-4 as signal transduction components [37].

Another signaling pathway of IL-6 involves the GTP-binding protein Ras, which may also be involved in other cytokine systems. GTP-binding motifs are present in the gp130 intracytoplasmic region; however, their precise role is unclear [11]. This Ras-dependent pathway includes intermediate steps involving Raf, MEK (MAP kinase kinase), and MAPK [38–41]. Following translocation into the nucleus, it is believed that MAP kinase activates the nuclear factor for IL-6 (NF-IL6) transcription factor to act on its target genes [42]. The binding activity of NF-IL6 is most likely induced by IL-6 through the increased expression of the NF-IL6 gene, rather than through post-translational modification [43]. Other serine/threonine protein kinases can also be activated by IL-6 [44]. Moreover, IL-6 also activates phosphatidylinositol (PI3)-kinase through the activation of the p-85 subunit of PI3-kinase and contributes to the complexity of the cellular response to this cytokine [45–47].

3. IL-6 and prostate cancer

3.1. IL-6 and clinical prostate cancer

Multiple studies have demonstrated that IL-6 is elevated in the sera of patients with metastatic prostate cancer [48–50]. Adler et al. [48] demonstrated that serum levels of IL-6 and transforming growth factor-β1 are elevated in patients with metastatic prostate cancer, and that these levels correlate with tumor burden as assessed by serum PSA or clinically evident metastases. In a similar fashion, Drachenberg et al. [51] reported elevated serum IL-6 levels in men with hormone-refractory prostate cancer compared to normal controls, benign prostatic hyperplasia, prostatitis, and localized or recurrent disease. These observations suggest that IL-6 may be a surrogate marker of the androgen independent phenotype.

IL-6 has been shown to be a candidate mediator of prostate cancer morbidity, and a candidate marker of disease activity for prospective clinical testing [50]. Signs of morbidity associated with human prostate cancer include anorexia, anemia, cachexia, asthenia, elevated acute phase proteins, hypoalbuminemia, edema, anergy, and diffuse bone pain [50], and unlike most solid tumors, prostate cancer can cause death without causing massive destruction to vital organs by space occupying metastatic lesions [52]. Twillie et al. hypothesized that death in some patients with advanced hormone refractory prostate cancer may be caused by, or hastened by circulatory exposure of ejaculate proteins which have a normal role in the genitourinary tract, but are pathologic when they enter the systemic circulation in large amounts [50]. IL-6 has been shown to be a mediator of experimental cachexia [53], is a well documented mediator of inflammation, and elevated levels of this cytokine have been associated with morbidity in a number of chronic diseases [53]. In their 1995 study, Twillie et al. demonstrated that IL-6 is a normal component of seminal plasma, and that elevated serum levels of IL-6 in prostate cancer patients were associated with certain clinical parameters of morbidity such as leukocytosis, anemia, hypercholesterolemia, and elevated serum lactate dehydrogenase levels. In addition to its role as a mediator of morbidity, IL-6 may also act as a growth factor, and protect prostate cancer cells from cell death induced by certain chemotherapeutic agents. IL-6 has been implicated in the modulation of growth and differentiation in many malignant tumors and is associated with poor prognosis in several solid and hematopoietic neoplasms such as renal cell carcinoma, ovarian cancer, lymphoma, and melanoma [54]. Thus, taken together, these data provide a large body of evidence that IL-6 is associated with prostate cancer in the clinical arena.

3.2. Identification of IL-6 and IL-6R in human tissue samples

IL-6 is produced by a large number of cells. However, its secretion is generally tightly controlled, and serum levels in healthy individuals are very low [51]. In terms of prostatic disease, it has been demonstrated that normal prostate epithelial cells, cells derived from benign prostatic hyperplasia, and primary prostate tumors secrete IL-6 in cell culture [50]. Thus, the prostate cancer cells themselves may be the source of elevated IL-6 levels found in prostate cancer patients. In addition to IL-6, the IL-6R has been identified in human prostate carcinoma and benign prostatic hyperplasia [55]. Using slot-blot analysis with a probe that recognizes mRNA encoding the α-subunit, IL-6R expression was found in 78% of BPH and 100% of prostate carcinoma samples analyzed. Hobisch and Culig

demonstrated the expression of IL-6 and its receptor (Fig. 1) in malignant and normal prostate using immunohistochemistry [56]. In normal prostatic tissue, IL-6 immunoreactivity was predominantly confined to basal cells. In contrast, only a small percentage of glandular cells showed a positive immune reaction for this cytokine. Interestingly, there was no IL-6 reaction in stromal cells. However, IL-6 secretion was detected in the supernatants of prostatic fibroblasts and smooth muscle [56]. Based on these results, it was proposed that there is a high secretion rate of IL-6 in prostatic stromal cells and therefore it is not detectable in these cells by immunohistochemistry. An alternative explanation is that process of cell culture itself stimulated IL-6 expression in the primary cells cultures, thus resulting in secretion of IL-6 in the supernatant. IL-6 staining pattern changed in high-grade prostate intraepithelial neoplasia (PIN) lesions and in cancer tissue. In 11 of 23 Gleason patterns investigated, there were more than 50% of IL-6-positive tumor cells. This study revealed that the IL-6 receptor is expressed in normal prostate, high-grade PIN and cancer tissue [56]. In nonmalignant tissue, IL-6R was detected in both basal and secretory cells. All prostate cancers examined in the study, including those that were poorly differentiated, stained positive for IL-6R [56]. One possible mechanism to account for this observation is that IL-6 affects prostatic growth and function in an autocrine and paracrine manner.

3.3. *Effects of IL-6 on human prostate cancer cell lines*

Studies of IL-6 expression in prostate cancer were initially carried out in a variety of prostate cancer cell lines. The androgen-refractory cell lines PC-3, DU145, and TSU secrete a number of cytokines including high levels of IL-6 [57]. IL-6 was also detected in the supernatants of cultured prostatic stromal and epithelial cells [50,56,58]. There have been conflicting reports as to whether or not the androgen-responsive cell line LNCaP secretes IL-6. We and others have observed IL-6 production in this cell line [4,54] while other investigators have reported minimal or no IL-6 production by LNCaP cells [57,59]. We have observed that phorbol esters induce IL-6 production from LNCaP cells [60]. Okamoto et al. showed that the addition of dihydrotestosterone (DHT) to the culture medium of LNCaP cells could stimulate the production of IL-6 in a dose-dependent manner [59]. In contrast, we have demonstrated that DHT inhibits phorbol-ester-induced IL-6 production from LNCaP cells [60]. These results are in agreement with other investigators that have demonstrated androgens inhibit IL-6 production in bone marrow stromal cells [61].

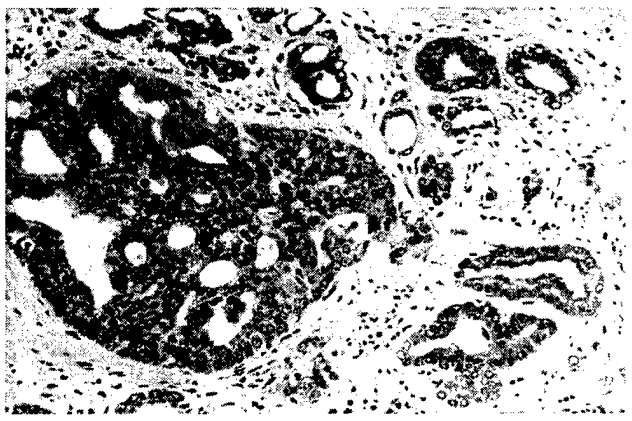

Fig. 1. Immunohistochemical staining for IL-6 receptor on paraffin-embedded adenocarcinoma tissue. The majority of cells express IL-6 receptor (reproduced from Hobisch et al. [56] with permission of John Wiley and Sons).

In contrast to the conflicting data regarding IL-6, the presence of IL-6 receptor is more consistent. Specifically, the hormone refractory cell lines DU-145, PC-3 and TSU, and the hormone-dependent cell lines LNCaP, LNCaP-ATCC, and LNCaP-GW have been shown to express both components of the IL-6 receptor complex. The presence of IL-6 receptor in these cells was first demonstrated with the use of a chimeric toxin, composed of IL-6 and Pseudomonas endotoxin that selectively bound to IL-6 receptor and resulted in cell death [54]. Further studies identified the ligand binding subunit (IL6Rp80) by slot blot analysis [55], ELISA and RT-PCR [57], and the signal transduction subunit (gp130) by sequential immunoprecipitation and immunoblotting [57]. Proliferation studies carried out with prostate cancer cell lines revealed different effects of IL-6 on androgen-sensitive and insensitive cells [57]. Just as there have been contrasting results regarding the production of IL-6 by LNCaP cells, a number of studies from various laboratories have yielded contrasting results regarding the effects of IL-6 on the growth of these cells. Chung et al. showed that inhibition of IL-6 resulted in decreased cell growth of hormone-refractory cells, but had no effect on the growth of hormone-dependent cell lines [57]. Addition of exogenous IL-6 to the culture media of LNCaP cells by several groups has resulted in a dose-dependent inhibition of cell growth [57,58,62–65]. On the other hand, some researchers observed a stimulatoy response after treatment with IL-6 [66,67]. The reasons for these differences have not been clarified to date but it seems that IL-6 in human prostate cancers exerts divergent effects and therefore it will be interesting to learn more about its co-localization with molecules that regulate cellular proliferation. It thus appears that IL-6 acts as an autocrine and paracrine growth factor in PC-3, TSU, and DU145 cells [57], and as a paracrine growth inhibitor in LNCaP cells [57,58]. However, in the presence of androgen, IL-6 acts as an autocrine growth

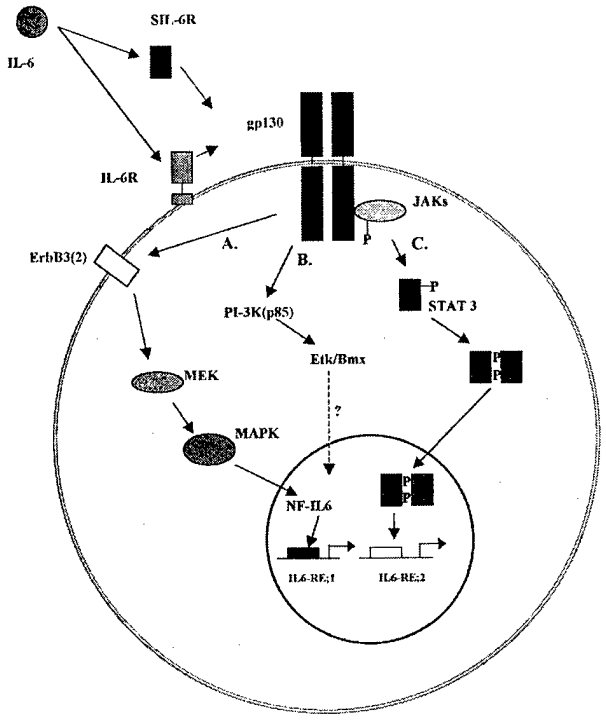

Fig. 2. IL-6 signaling in prostate cancer cells. IL-6 signaling through gp130 initiates three pathways proposed to occur in prostate cancer. (A) IL-6-induced association of gp130 and ErbB3 or ErbB2 leads to activation of signaling through the MAP kinase pathway with subsequent activation of NF-IL-6. Once activated, NF-IL-6 binds to IL-6 response element type one (IL6-RE;1) resulting in gene transcription, (B) IL-6 promotes co-precipitation of gp130 with the p85 subunit of PI3-kinase (PI3-K). PI3-K is involved in Etk/Bmx activation, which leads to nuclear translocation of unidentified transcription factors that activate transcription, (C) IL-6 signaling is primarily mediated through the activation of the JAK/STAT pathway. Homodimerization of gp-130 (following IL-6/IL-6R binding) activates associated tyrosine kinases that subsequently cause gp130 phosphorylation (P). Activated JAK phosphorylates STAT3 which homodimerizes and translocates to the nucleus. Once in the nucleus, this STAT3 homodimer binds to the type 2 IL-6 response element (IL6-RE;2) which initiates transcription.

factor in LNCaP cells [59]. Applying these findings to behavior of prostate cancer in vivo, Chung et al. have suggested that IL-6 may undergo a functional transition from paracrine growth inhibitor to autocrine growth stimulator during the progression of CaP to the hormone refractory phenotype [57].

3.4. IL-6 signaling in prostate cancer

IL-6 mediates its affects on prostate cancer cells through a variety of signal transduction pathways (summarized in Fig. 2). For example, IL-6 activates the PI3-kinase cascade in prostate cancer cells, resulting in differentiation [68] and inhibition of apoptosis [69]. Qiu et al. demonstrated that PI3-kinase is involved in Etk activation based on the following findings: (i) Wortmannin, a specific inhibitor or PI3-kinase, abolished the activation of Etk by IL-6, (ii) a constituitively active p110 subunit of PI3-kinase was able to activate Etk in the absence of IL-6, and (iii) a dominant negative p85 subunit of PI3-kinase mutant blocked the activation of Etk by IL-6 [68]. Consequently, IL-6 treatment of LNCaP induced a neuroendocrine-like differentiation phenotype, with neurite extension and enhanced expression of neuronal markers. The phenotype could be abrogated by the overexpression of a dominant-negative Etk, indicating Etk is required for this differentiation process. In Chung et al.'s study [69], tyrosine phosphorylation of p85 was upregulated by IL-6 in both LNCaP and PC-3 cells. IL-6 promoted co-precipitation of p85 with gp130. Inhibition of PI3-kinase with wortmannin induced programmed cell death in PC-3 cells. Both lines of investigation indicated the participation of PI3-kinase in the IL-6 pathway. Qiu et al. also discovered that binding of IL-6 in LNCaP cells induced the association of gp130 with growth factor receptors (ErbB2, ErbB3), and subsequently leads to activation of the MAP kinase pathway [66].

In general, IL-6 signaling is primarily mediated through the activation of JAK/STAT pathway. Lou et al. showed that IL-6 stimulates prostate cancer growth through this pathway [70]. Another study reports that STAT3 transcriptional activation correlates with the growth-inhibitory signal of IL-6 in LNCaP cells, suggesting that STAT3 transcriptional activity is an important determinant in the different phenotypic responses to IL-6 in prostate cancer [71]. Moreover, Chen et al. reported that IL-6-induced activation of STAT3 in LNCaP cells increased AR-mediated gene activation. In particular, they showed that STAT3 associated with the AR in an androgen-independent, but IL-6 dependent, manner [72]. This reveals the importance of IL-6 signaling to induce AR-mediated gene activation in prostate carcinoma cells and the importance of activated STAT3 in human tumor development and progression.

3.5. IL-6 and the androgen receptor

The AR, which is expressed in normal prostate tissue and heterogeneously in prostate cancers, is a key transcription factor in the prostate [73–76]. Activation of the AR in prostate cancer is being intensively investigated, and there is evidence that the AR could be stimulated by a number of nonsteroidal compounds, such as polypeptide growth factors, protein kinase A activators, vitamin D and neuropeptides [77–81]. IL-6 activates the AR in a ligand-independent manner and induces a synergistic AR response with very low concentrations of androgen [62,72]. Our previous report that androgen inhibits AR activity [60] combined with the observation that IL-6 activates AR demonstrates that cross talk between IL-6 and AR exists. The interaction between IL-6 and AR might be particularly

important in patients with advanced prostate cancer who have elevated serum levels of IL-6 [48–50].

3.6. IL-6 and chemotherapy

Defining mechanisms to control IL-6 or IL-6R expression may prove useful for therapy of the many clinical disorders in which IL-6 plays a role [4]. Addition of anti-IL-6 antibody to the growth medium of the hormone independent cell lines DU145, PC-3 [57,82], and TSU [57] inhibits cell growth. In vitro studies have shown that the addition of anti-IL-6 enhances the cytotoxicity of certain chemotherapeutic agents in PC-3 cells, which are resistant to the drugs alone [82]. Borsellino et al. later showed that the activity of IL-6 was more efficiently blocked with an IL-6R antagonist, Sant7, and that this too potentiated the sensitivity of PC-3 cells to etoposide-mediated cytotoxicity [83]. In this study, the investigators also blocked signaling through gp130 in PC-3 using a gp130 antisense oligodeoxynucleotide. This inhibited cell growth and viability by about 20% and increased sensitivity to etoposide, confirming the positive role of endogenous IL-6 in cell survival [83]. Furthermore, Borsellino et al. showed that gp130-mediated signaling does not influence or minimally influences the bcl-2 mediated antiapoptotic pathway and that, rather, it proceeds through a *ras*-dependent pathway [83]. These data suggest that endogenous IL-6 acts to protect tumor cells from drug-induced cell death, and its neutralization may be a useful adjuvant to chemotherapy.

4. Conclusion

Clinical studies of prostate cancer patients have implicated IL-6 as a potential mediator of prostate cancer morbidity and as a marker for advanced hormone-refractory prostate cancer. The various functions of IL-6 may allow it to play multiple roles in the pathophysiology of prostate cancer. It has been shown to act as an autocrine and paracrine growth factor in hormone refractory human prostate cancer cell lines and as a paracrine growth inhibitor in hormone dependent cell lines. These data suggest that IL-6 plays a role in the transition of prostate cancer from a hormone-dependent to a hormone-refractory phenotype. The ability of IL-6 to mediate signaling through the AR in the absence of androgen also lends credence to this hypothesis. In vitro studies have also shown that IL-6 plays a key role in protecting prostate cancer cells from chemotherapeutic-mediated cell death. Some of the signaling pathways through which IL-6 mediates its effects have been defined, giving us a better understanding of how IL-6 contributes to the pathophysiology of the disease. Taken together, these studies suggest that the inhibition of IL-6 may be a useful adjunct to prostate cancer therapy as in vitro data have shown, and could decrease morbidity in patients with advanced disease.

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