

Interleukin-6 Promotes Androgen-independent Growth in LNCaP Human Prostate Cancer Cells¹

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ABSTRACT

Purpose: Prostate cancer frequently progresses from an initial androgen dependence to androgen independence, rendering the only effective androgen ablation therapy useless. The mechanism underlying the androgen-independent progression is incompletely understood. Interleukin (IL)-6 has been implicated in this androgen-independent progression. In this study, we tested whether IL-6 induces androgen-independent growth both *in vitro* and *in vivo*.

Experimental Design: IL-6 was expressed in androgen-sensitive LNCaP cells. The effects of IL-6 on androgen receptor activity was determined by Northern blots and gel shift assays. The effects of IL-6 on LNCaP cell growth were determined *in vitro* by MTT assay and *in vivo*.

Results: IL-6 can enhance the growth of androgen-sensitive LNCaP cells in the androgen-deprived condition *in vitro*, which is accompanied by elevation of androgen-regulated prostate-specific antigen mRNA expression. IL-6 promotes androgen-sensitive LNCaP cell tumor growth in the castrated male mice. IL-6 enhances androgen receptor DNA binding activity and nuclear translocation. The androgen-independent phenotype induced by IL-6 in LNCaP cells is accompanied by significant activation of signal transducers and activators of transcription 3 and mitogen-activated protein kinase signal pathways.

Conclusions: These studies clearly provide experimental evidence that IL-6 initiates and/or enhances the transition of prostate cancer cells from an androgen-dependent to an androgen-independent phenotype.

INTRODUCTION

The growth of prostate epithelial cells requires a physiological level of androgen, both to stimulate proliferation and inhibit apoptotic death (1). Androgen binds to the AR,⁴ which triggers interaction of AR to specific AREs in the promoters of androgen-regulated genes. These interactions facilitate the activation or repression of genes regulating development, differentiation, and proliferation of prostate epithelial cells. Currently, the standard treatment for metastatic prostate cancer is androgen ablation therapy. The problem is that whereas almost all patients with prostate cancer initially respond to androgen ablation therapy, virtually every patient will relapse to hormone-refractory disease due to the growth of androgen-independent cancer cells, rendering the only effective therapy useless. The molecular cause of acquired androgen-independent growth, which is promoted by activation of AR signaling through AR gene mutation and amplification (2, 3), coactivators (4), and cross-talk between the AR and protein kinase pathways (4, 5), is incompletely understood. There is growing evidence that suggests growth factors and cytokines play an important role in acquisition of hormone independence.

IL-6 is a glycoprotein consisting of 212 amino acids encoded by the IL-6 gene localized to chromosome 7p21–14 (6). IL-6 is a pleiotropic cytokine that plays a central role in host defense mechanisms by regulating immune responses, hematopoiesis, and the induction of acute phase reaction (6). The biological activities of IL-6 are mediated by the IL-6 receptor. The receptor for the IL-6 family of cytokines (IL-6, IL-11, ciliary neurotrophic factor, oncostatin M, and leukemia inhibitory factor) is composed of an IL-6-specific receptor subunit (α chain) and a signal transducer, gp130 [β chain (7)]. The binding of IL-6 to its receptor resulted in activation of intracellular signaling including Janus kinase-Stat and MAPK pathways (7, 8).

The expression and function of IL-6 in prostate cancer have been the subject of multiple recent studies. The expression of IL-6 and its receptor has been consistently demonstrated not only in human prostate cancer cell lines but more importantly in human prostate carcinoma and benign prostate hyperplasia obtained directly from patients (9–11). The levels of IL-6 in serum are significantly elevated in many men with advanced, hormone-refractory prostate cancer (12, 13). Furthermore, IL-6

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⁴The abbreviations used are: IL, interleukin; AR, androgen receptor; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PSA, prostate-specific antigen; Stat, signal transducers and activators of transcription; MAPK, mitogen-activated protein kinase; ARE, androgen-responsive element; FBS, fetal bovine serum; CS, charcoal-stripped; RT-PCR, reverse transcription-PCR; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; EMSA, electrophoretic mobility shift analysis; PI3K, phosphatidylinositol 3'-kinase; ERK, extracellular signal-regulated kinase.

has been demonstrated as a candidate mediator of human prostate cancer morbidity (14). IL-6 has been suggested to have both growth-promoting and -inhibiting activities in androgen-dependent LNCaP human prostate cancer cells *in vitro*. IL-6 can function as a paracrine growth factor for the human LNCaP androgen-sensitive prostate cancer cells and an autocrine growth factor for human DU145 and PC3 androgen-insensitive prostate cancer cells (15–18). IL-6 can also function as a paracrine growth inhibitor for LNCaP cells and an autocrine growth stimulator for the DU145 and PC3 cells (19). Recently, results from a number of groups demonstrated that IL-6 activates AR-mediated gene expression in LNCaP cells *in vitro* (17, 20–22), suggesting that IL-6 may play a critical role during the progression of prostate cancer.

Whereas numerous studies have suggested the role of IL-6 in the growth and androgen responsiveness of prostate cancer cells *in vitro*, there is no experimental evidence to demonstrate the role of IL-6 in the promotion of androgen-independent growth of prostate cancer cells *in vivo*. In this study, we tested whether IL-6 induces androgen-independent growth. We demonstrate that IL-6 induces androgen-independent growth of androgen-sensitive LNCaP human prostate cancer cells both *in vitro* and *in vivo*, which is accompanied by elevation of PSA levels. The androgen-independent phenotype induced by IL-6 in LNCaP cells is mediated in large part by activation of Stat3 signaling and potentially also by activation of the MAPK pathway.

MATERIALS AND METHODS

Cell Culture. The LNCaP cells were maintained in RPMI 1640 containing penicillin (100 units/ml), streptomycin (100 µg/ml), and 10% FBS at 37°C in 5% CO₂ incubator. The IL-6-overexpressing cells (LN-S15 and LN-S17) and neo control (transfected with vector alone) cells were cultured in the same medium plus 0.3 mg/ml G418. To investigate the androgen withdrawal effect, cells were cultured in medium containing 10% CS-FBS instead of regular 10% FBS.

In Vitro Cell Proliferation. LNCaP cells or IL-6-overexpressing cells (LN-S15 and LN-S17; 10⁴ cells/well) were plated in 12-well plates in RPMI 1640 containing 10% FBS. After 2 or 3 days in regular culture medium with 10% FBS, cells were switched into a medium of phenol red-free RPMI 1640 containing either 10% FBS or 10% CS-FBS (Hyclone). For controls, antihuman IL-6 antibody (20 µg/ml; Sigma) was added into the tissue culture medium. Two days later, cells were determined by using the MTT assay (Sigma) according to the manufacturer's instructions.

In Vivo Tumor Growth. Four- to six-week-old athymic male nude mice (Harlan, Indianapolis, IN) were inoculated s.c. in the flank with 3 × 10⁶ cells (LNCaP, neo, LN-S15, and LN-S17) resuspended in Matrigel (BD Biosciences, Bedford, MA) diluted 1:1 in complete culture medium. The volume of the growing tumors was estimated by measuring three tumor dimensions (length × width × depth) with a caliper (23).

RT-PCR. RT-PCR was performed as follows. Briefly, total RNA was isolated from cells using the Trizol method (Life Technologies, Inc., Rockville, MD). One µg of total RNA was used in the reverse transcription reaction, and thermal cycling

was programmed as follows: 1 min at 4°C; 2 min at 70°C; and 5 min at 4°C with oligodeoxythymidylic acid. After chilling tubes on ice, buffer, deoxynucleotide triphosphates, RNase inhibitor, and mouse mammary tumor virus were added and incubated at 42°C for 1 h. The cDNAs thus obtained were amplified with 30 cycles (45 s at 95°C, 1 min at 58°C, and 1 min at 72°C) of PCR reaction in the presence of Taq polymerase (Promega, Madison, WI). PSA primer sequences used were 5'-GGCAGGTGCTTGTAGCCTCTC-3' (sense) and 5'-CAC-CCGAGCAGGTGCTTTTGC-3' (antisense). The PCR products were then resolved in a 1.5% agarose gel, and bands were analyzed with Molecular Imager FX System (Bio-Rad, Hercules, CA). GAPDH primers were used as control.

Northern Blot. Twenty µg of RNAs were electrophoresed in 1.2% denaturing agarose gels and transferred to a nylon membrane (MSI, Westborough, MA). A 1.1-kb *Bam*HI fragment containing the PSA cDNA was labeled with [α -³²P]dCTP (3000 Ci/mmol; ICN, Costa Mesa, CA) using Ready-To-Go DNA Labeling Beads (Amersham Pharmacia Biotech, Buckinghamshire, United Kingdom). Hybridization was carried out during 3 h at 65°C in Rapid-hyb buffer (Amersham Pharmacia Biotech). Membranes were washed for 15 min at 65°C in 2× SSC, 0.1% SDS (twice); 0.5× SSC, 0.1% SDS; and 0.1× SSC, 0.1% SDS. Radioactivity in the membranes was analyzed with a Molecular Imager FX System (Bio-Rad).

Determination of PSA Secretion. The serum was collected at the end of experiments. Fifty µl of serum were used to determine PSA secretion. Levels of PSA in the serum of tumor-bearing mice were determined by ELISA with the use of anti-PSA as primary antibody as described by the manufacturer's protocol (Bechman Coulter, Fullerton, CA).

EMSA. Whole cell extracts were prepared by using high-salt buffer [20 mM HEPES (pH 7.9), 20 mM NaF, 1 mM Na₂P₂O₇, 1 mM Na₃VO₄, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 0.5 mM phenylmethylsulfonyl fluoride, 420 mM NaCl, 20% glycerol, 1 µg/ml leupeptin, and 1 µg/ml aprotinin], followed by snap-freezing in ethanol/dry ice for 5 min and thawing on ice for 10 min. The freeze and thaw procedures were repeated again for a total of two times. The supernatant was then centrifuged and harvested. Protein concentrations were determined by Coomassie Blue plus protein assay kit (Pierce) according to the manufacturer's protocol. Stat3 DNA binding activity was determined by EMSA using Stat3 consensus oligonucleotide 5'-GATCCTTCTGGGAATTCCTAGATC as described previously (24). For determination of the AR DNA binding activity, whole cell extracts (20 µg) were incubated in a final volume of 20 µl [10 mM HEPES (pH 7.9), 80 mM NaCl, 10% glycerol, 1 mM DTT, 1 mM EDTA, 100 µg/ml poly(deoxyinosinic-deoxycytidylic acid)] by EMSA with radiolabeled double-stranded AR consensus binding motif (Santa Cruz Biotechnologies, Santa Cruz, CA). The protein-DNA complexes were resolved on a 4.5% nondenaturing polyacrylamide gel containing 2.5% glycerol in 0.25× Tris-borate EDTA at room temperature, and the results were autoradiographed. Quantitation of the amount of AR DNA binding activity in the "protein-DNA" bandshift was measured using the Molecular Imager FX System (Bio-Rad). For the supershift experiment, 20 µg of cell extracts were incubated with either Stat3 antibody or AR antibody

(Santa Cruz Biotechnologies) for 1 h at 4°C before incubation with the radiolabeled probe.

Nuclear Lysate Preparation. Nuclear protein extracts were prepared as described previously (17). Briefly, for nuclei preparation, cells were harvested, washed with PBS twice, resuspended in hypotonic buffer [10 mM HEPES-KOH (pH 7.9), 1.5 mM MgCl₂, 10 mM KCl, and 0.1% NP40], and incubated on ice for 10 min. Nuclei were precipitated with 3,000 × *g* centrifugation at 4°C for 10 min. After washing once with hypotonic buffer, the nuclei were lysed in lysis buffer [50 mM Tris-HCl (pH 8.0), 150 mM NaCl, and 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 determined by Coomassie Blue plus protein assay kit.

Western Blot Analysis. Forty μg of protein were resolved in 8–12% SDS-PAGE, depending on the molecular weight of the protein to be detected. After blocking overnight at 4°C in 5% milk in PBS-0.1% Tween 20, membranes were incubated overnight with antibodies against either Stat3, phosphorylated Stat3, p44/42ERK1/2, phosphorylated Akt (Cell Signaling Technology) or AR (Santa Cruz Biotechnology). After secondary antibody incubation, immunoreactive proteins were visualized with an enhanced chemiluminescence detection system (Amersham Pharmacia Biotech).

RESULTS

Effect of IL-6 on the Growth of LNCaP Cells *in Vitro*.

It was demonstrated previously that the androgen-sensitive LNCaP cells express IL-6 receptor but express no detectable IL-6 protein (15–19). IL-6 can enhance AR-mediated PSA expression in LNCaP cells (17, 18, 20–22), suggesting that IL-6 can enhance androgen responsiveness of LNCaP cells. To determine the effect of IL-6 on the growth of LNCaP cells in the presence and absence of androgen, we ectopically expressed IL-6 by introduction of a full-length IL-6 cDNA into IL-6-negative LNCaP cells as described previously (16). Several stable transfectants containing IL-6 cDNA in the sense orientation and vector-alone controls were selected in the presence of G418, subcloned, and tested for their expression of IL-6 by ELISAs. Two stable IL-6 transfectants (LN-S15 and LN-S17) expressing high levels of IL-6 (2465 and 2743 pg/ml/10⁶ cells, respectively) were selected for additional studies. To test whether IL-6 promotes LNCaP androgen-independent cell growth *in vitro*, parental LNCaP, neo control, and IL-6-overexpressing clones were cultured in the presence and absence of androgen, and the cell growth was determined. As shown in Fig. 1A, the growth of androgen-sensitive LNCaP cells and neo control in culture was reduced by about 50% after 48 h in androgen-deprived CS serum (Hyclone; testosterone concentration is <10⁻¹¹ M, in which prostate epithelial cells do not respond to testosterone stimulation; Ref. 25) compared with that in normal serum (testosterone concentration is about 10⁻⁹ M). Addition of dihydrotestosterone (10⁻⁹ M) in the CS serum restored LNCaP cell growth to levels similar to that of the complete normal serum (data not shown). In the clones of LNCaP cells overexpressing IL-6 (LN-15 and LN-17), however, there was only a 5–10% decrease in growth under these andro-

gen-deprived conditions compared with growth in normal serum, suggesting that overexpression of IL-6 can enhance the growth of LNCaP cells in the androgen-deprived condition *in vitro*. Addition of anti-IL-6 antibody in IL-6 overexpression clones restored the growth inhibition to about 60% under androgen-deprived conditions compared with growth in normal serum.

IL-6 Induces Androgen-independent Growth *in Vivo*.

Having demonstrated that IL-6 enhances the growth of androgen-sensitive LNCaP cells in the absence of androgen *in vitro*, we further tested the effect of overexpression of IL-6 on the growth of androgen-sensitive LNCaP human prostate cancer cells *in vivo*. To test the effects of IL-6 on tumor formation and induction of androgen-independent growth of LNCaP cells *in vivo*, 8-week-old male nude mice were randomly divided into two groups; one group was left intact, and the other group received surgical castration, in which the residual levels of androgen are insufficient to maintain growth of androgen-sensitive LNCaP cells (1). Three days after castration, intact or castrated male nude mice were inoculated s.c. with parental LNCaP, vector control neo, or LNCaP cell clones overexpressing IL-6 with coinoculation of Matrigel. For the two independent IL-6-overexpressing clones, tumors became apparent at the site of injection within 30 days in the intact male mice and within 40 days in the castrated male mice (Fig. 1, B and C). Parental LNCaP cells and vector control neo clone did not grow any detectable tumor in both intact (within a 40-day observation period) and castrated (within a 70-day observation period) male nude mice. These results demonstrate that IL-6 promotes the growth of androgen-sensitive LNCaP cells in the absence of androgen *in vivo*.

IL-6 Enhances Androgen-responsive Gene PSA Expression *in Vitro* and *in Vivo*.

Results from a number of groups demonstrated that IL-6 activates AR-mediated PSA gene expression in LNCaP cells *in vitro* (17, 20–22). To test whether overexpression of IL-6 enhances the expression of an endogenous, androgen-regulated PSA, the expression of PSA was compared between the parental and IL-6-overexpressing LNCaP cells in the presence and absence of androgen. As shown in the Fig. 2A, in the presence of androgen, PSA mRNA expression was elevated in the IL-6-overexpressing LNCaP cells compared with the parental and vector control LNCaP cells. When the cells were cultured in phenol red-free medium supplemented with the CS serum, in which the androgen was deprived, PSA mRNA expression was elevated in the IL-6-overexpressing clones compared with the parental and vector control LNCaP cells (Fig. 2B), suggesting that overexpression of IL-6 can enhance endogenous PSA expression in the presence and absence of androgen. These results are consistent with previous reports that IL-6 activates the PSA promoter/enhancer in the presence and absence of androgen (17, 20, 21). In addition, tumors generated from IL-6-overexpressing LNCaP cells also produced high levels of circulating PSA in the serum of both the intact male mice (average, 38 ng/ml per g of tumor) and the castrated male mice (average, 32 ng/ml per g of tumor).

To test whether IL-6 can influence the DNA binding activity of AR protein to the ARE, we performed EMSA using radiolabeled oligonucleotides of the ARE with nuclear extracts from LNCaP cell clones. LNCaP clones overexpressing IL-6

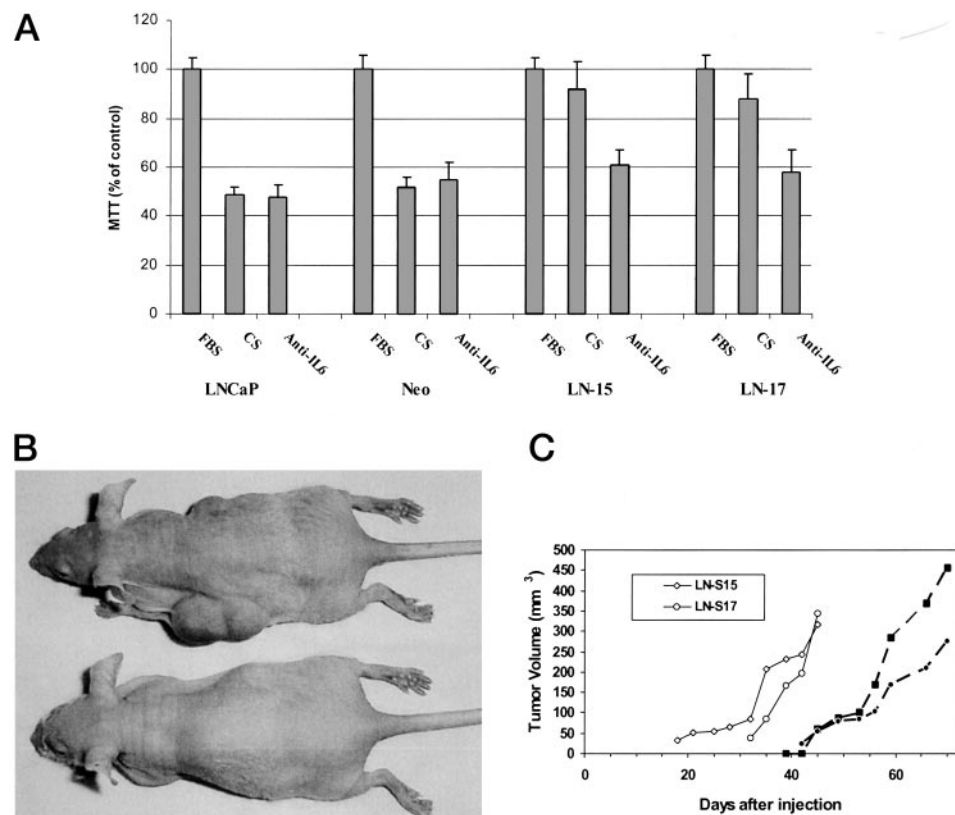


Fig. 1 Overexpression of IL-6 promotes LNCaP androgen-independent growth. **A**, effect of overexpression of IL-6 on LNCaP cell growth in the presence and absence of androgen *in vitro*. Cells were cultured in RPMI 1640 supplemented with 10% FBS. After 24 h, the cells were switched to either 10% FBS or 10% CS-FBS (CS). For controls, cells were cultured in RPMI 1640 supplemented with 10% CS-FBS plus 20 μ g/ml anti-IL-6 antibody. After incubation for another 72 h, MTT assays were performed. MTT values for the complete FBS were expressed as 100%, and MTT values for CS-FBS were expressed as a percentage relative to complete FBS. **B**, IL-6-overexpressing clone (LN-15) developed tumors *versus* parental LNCaP cells that did not grow any tumor in the castrated male nude mice. **C**, tumor growth curve in the intact and castrated male nude mice. Parental LNCaP cells and neo clone or IL-6-expressing clones (LN-15 and LN-17) were injected into the intact (solid lines, open symbols) or castrated (broken lines, filled symbols) male nude mice ($n = 8$ for each condition). Parental LNCaP and neo control did not grow tumor.

showed an increase in AR-ARE complex formation compared with the parental LNCaP cells in the presence of androgen as well as in the absence of androgen (Fig. 3A). The AR-ARE complex in the IL-6-overexpressing clones has little change in the absence of androgen compared with that in the presence of androgen (Fig. 3A). The specificity of this AR-ARE complex was demonstrated by supershift assay using antibody specifically against AR (Fig. 3B).

The AR typically translocates to the nucleus to exert its function on gene expression. To examine whether overexpression of IL-6 affects the expression and translocation of AR, Western blot analysis was performed using cell extracts from either whole cell extracts or nuclear extracts. As shown in Fig. 3C, overexpression of IL-6 in LNCaP cells significantly enhanced the expression of AR in the nuclear compartment without alteration of the total AR expression (whole cell extracts) both in the presence of androgen (FBS) and in the absence of androgen (CS-FBS).

Overexpression of IL-6 Activates Its Downstream Signaling Pathways in LNCaP Cells. The effects of IL-6 on prostate cancer cells are mediated by a variety of signal

transduction pathways including Janus kinase-Stat, MAPK, and PI3K-AKT pathways, resulting in proliferation, differentiation, and inhibition of apoptosis. To examine which pathways were altered by overexpression of IL-6 in LNCaP cells, cell lysis from parental and IL-6-overexpressing LNCaP cells were analyzed. We first examined the effect of overexpression of IL-6 on the expression and activation of Stat3, a major mediator of IL-6 signaling. As shown in Fig. 4A and 4B, overexpression of IL-6 significantly elevates the activity of Stat3 both in the presence of androgen (FBS) and in the absence of androgen (CS-FBS).

To determine whether the increased Stat3 activity is associated with increased Stat3 protein expression and elevated phosphorylation, Western blots of whole cell extracts from the parental LNCaP and IL-6-overexpressing clones were performed using antibodies specific against either phosphotyrosine Stat3 (Tyr-705) or total Stat3 protein. As shown in Fig. 4C, overexpression of IL-6 induces Stat3 phosphorylation in LNCaP cells without alteration of total Stat3 expression, which is consistent with the results shown that IL-6 induces Stat3 activation in LNCaP cells. Collectively, these results demonstrate that

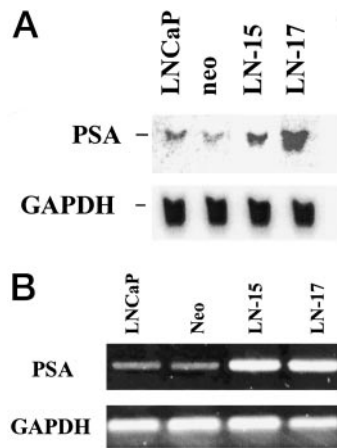


Fig. 2 Overexpression of IL-6 increases PSA mRNA expression in LNCaP cells. **A**, PSA mRNA expression in IL-6-overexpressing clones (LN-15 and LN-17), vector control (neo), and LNCaP cells cultured in normal FBS media and examined by Northern blot analysis using 20 μ g of total RNA. GAPDH is a control for equal loading. **B**, PSA mRNA expression in IL-6-overexpression clones (LN-15 and LN17), vector control (neo), and LNCaP cells cultured in androgen-deprived CS-FBS media for 72 h. The PSA mRNA was examined by RT-PCR. GAPDH is a control for equal loading.

overexpression of IL-6 significantly elevates Stat3 signaling in androgen-dependent human LNCaP prostate cancer cells.

To investigate whether overexpression of IL-6 alters Akt or MAPK signaling pathways in LNCaP cells, we performed Western blot analysis on cell extracts from parental LNCaP and IL-6-overexpressing clones using antibodies that specifically recognize either phosphorylated Akt or phosphorylated MAPK (p44/42ERK1/2), respectively. As shown in Fig. 4D, overexpression of IL-6 in LNCaP cells enhances the levels of phosphorylated (active) p44/42 ERK1/2 expression without altering the expression of total p44/42 ERK1/2 in both the presence and absence of androgen, whereas overexpression of IL-6 in LNCaP cells has less effect on the expression of phosphorylated Akt or total Akt in both the presence and absence of androgen (Fig. 4D).

Collectively, these results indicate that IL-6-induced signaling in LNCaP cells is mediated primarily through Stat3 and MAPK signaling pathways.

DISCUSSION

In the present study, we provide experimental evidence that IL-6 plays an important role in the induction of androgen-independent growth of human prostate cancer cells. We demonstrate that overexpression of IL-6 in androgen-sensitive human LNCaP prostate cancer cells results in the conversion of androgen-independent growth of LNCaP cells both *in vitro* and *in vivo*. Overexpression of IL-6 also enhances endogenous PSA expression in LNCaP cells, consistent with previous reports that IL-6 increases AR-mediated gene activation (17, 20–22). In addition, we demonstrate that IL-6 signaling is primarily mediated through activation of the Stat3 and MAPK signaling pathways in LNCaP cells.

The potential role of IL-6 in the development and progres-

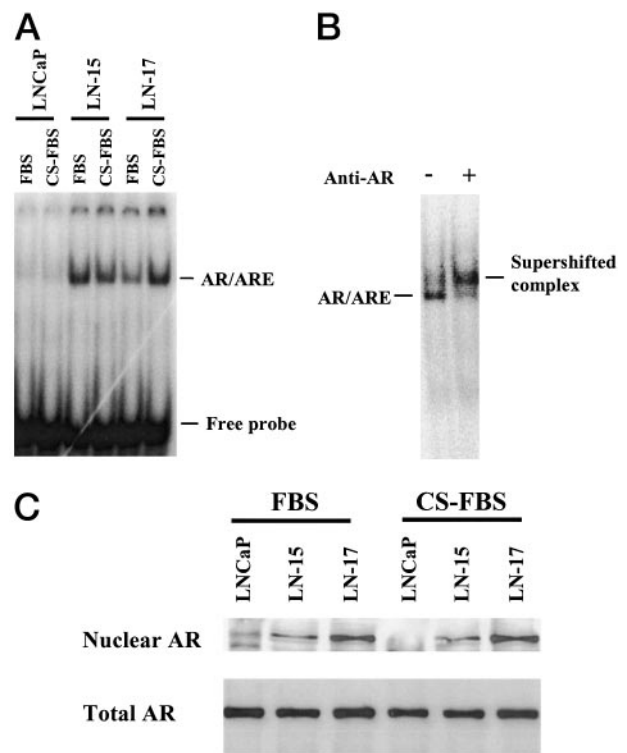


Fig. 3 Effect of overexpression of IL-6 on AR. **A**, IL-6 enhances the formation of AR-ARE complexes. EMSA was performed using radiolabeled ARE oligonucleotides with whole cell extracts isolated from LNCaP and IL-6-overexpressing clones (LN-15 and LN-17) cultured in FBS and androgen-deprived CS-FBS media. **B**, supershift assay of LN-17 cell extract using anti-AR antibody. Whole cell extracts were preincubated with antibodies specifically against AR as indicated. The positions of the AR-ARE and the supershifted complexes were indicated. **C**, overexpression of IL-6 enhances nuclear AR expression in LNCaP cells in the presence of androgen (FBS), but not in the absence of androgen (CS-FBS). Total cellular extracts and nuclear extracts were subjected to Western blot analysis (40 μ g/lane) using an antihuman AR antibody. The cells were cultured in RPMI 1640 with 10% FBS for 24 h and then switched to either 10% FBS or 10% CS-FBS, and culture continued for another 72 h. Cell lysis were extracted and used for the assays.

sion of prostate cancer cells has been suggested by numerous studies. Clinically, the levels of IL-6 in serum are significantly elevated in many men with advanced, hormone-refractory prostate cancer (12, 13). In addition, increased expression of IL-6 and IL-6 receptor has been demonstrated in prostate cancer tissues, and increased IL-6 receptor is correlated with increased proliferation of prostate cancer cells (9–11, 18). Experimentally, IL-6 has been suggested to have both growth-promoting and -inhibiting activities in androgen-dependent LNCaP human prostate cancer cells *in vitro* (16, 18, 19, 22). It has been demonstrated that IL-6 can act as a growth factor for both normal primary prostate epithelial cells and LNCaP prostate cancer cells *in vitro* (16–18). IL-6 stimulates prostate-specific protein expression in prostate carcinoma cells by activation of the AR and can be blocked by the antiandrogen bicalutamide (17, 20–22), consistent with our finding that overexpression of IL-6 enhances endogenous PSA expression in LNCaP cells. It

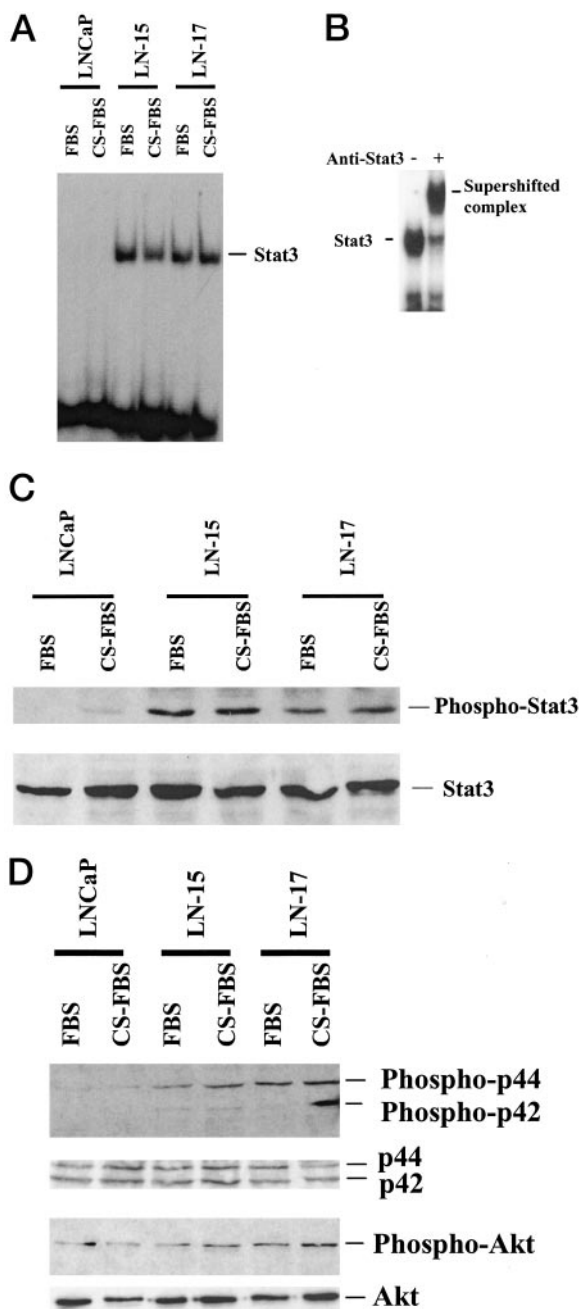


Fig. 4 Overexpression of IL-6 activates the Stat3 and MAPK pathways. **A**, IL-6 induces Stat3 activation in LNCaP cells. EMSA was performed using radiolabeled Stat3 oligonucleotides with whole cell extracts isolated from LNCaP and IL-6-overexpressing clones (LN-15 and LN-17) cultured in FBS and androgen-deprived CS-FBS media. **B**, supershift assay of LN-17 cell extract using anti-Stat3 antibody. Whole cell extracts were preincubated with antibodies specifically against Stat3 as indicated. The positions of Stat3 and the supershifted complexes were indicated. **C**, overexpression of IL-6 enhances Stat3 phosphorylation in LNCaP cells. Western blots were performed using antibodies against either phospho-specific Stat3 (Tyr-705) or total Stat3 with whole cell extracts isolated from LNCaP and IL-6-overexpressing clones (LN-15 and LN-17) cultured in FBS and androgen-deprived CS-FBS media. **D**, the effect of overexpression of IL-6 on Akt and MAPK expression in LNCaP cells. Whole cell extracts from parental LNCaP cells and IL-6-overexpressing clones (LN-15 and LN-17) cultured in either normal FBS or CS-FBS conditions were subjected to Western blot analysis

has also been indicated that IL-6 can mediate LNCaP cell growth arrest and induction of neuroendocrine differentiation (26, 27). Whereas all of the observed effects of IL-6 on the growth of prostate cancer cells were performed in tissue culture cells, mostly in androgen-dependent LNCaP human prostate cancer cells *in vitro*, the potential effects of IL-6 on LNCaP cells *in vivo* have not been reported. The present study is the first to provide such experimental evidence that IL-6 induces androgen-independent growth of androgen-sensitive human LNCaP prostate cancer cells both *in vitro* and *in vivo*. We have observed that overexpression of IL-6 in LNCaP cells significantly activates the Stat3 and MAPK signaling pathways. The observation of Stat3 activation by IL-6 is consistent with other reports that IL-6 stimulates prostate cancer cell growth through activation of the Stat3 signaling pathway (16, 18, 22, 27), and IL-6-induced activation of Stat3 in LNCaP cells increases AR-mediated gene activation in an androgen-independent but IL-6-dependent manner (20). IL-6 can activate erbB2 receptors, leading to activation of the MAPK pathway (22, 28). We also demonstrated that overexpression of IL-6 in LNCaP cells has less effect on the activation of Akt phosphorylation, which is different to the report that IL-6 can lead to activation of PI3K-Akt resulting in prevention of programmed cell death in human prostate cancer cell lines (18, 25, 29). The differential effects of IL-6 on the various signaling pathways (Stat3, MAPK, and PI3K-Akt) in LNCaP cells resulting in cell proliferation, differentiation, and survival are intriguing and are currently under intensive investigation.

PSA is a marker for prostate cancer, and the rise of the levels of PSA in the serum is an important indicator of prostate cancer progression. Several reports have indicated that IL-6 enhances PSA expression in LNCaP cells *in vitro* (17, 20–22), possibly through activation of Stat3 signaling (20). This is consistent with our finding that overexpression of IL-6 in LNCaP cells enhances endogenous PSA expression. In addition, we further demonstrated that overexpression of IL-6 induces PSA secretion to the serum in the castrated male nude mice, indicating that PSA levels induced by IL-6 are accompanied by LNCaP tumor growth in castrated male nude mice, similar to the clinical observation that rising PSA levels are a potential indicator of hormone-refractory prostate cancer. We have also demonstrated that overexpression of IL-6 enhances AR-ARE DNA binding activity and enhances AR nuclear translocation in LNCaP cells, which is consistent with the report that IL-6 increases AR expression in LNCaP cells (17).

One of the limitations of this study may be that IL-6 affects only LNCaP cells. LNCaP is an androgen-sensitive human prostate cancer cell line expressing a functional but mutant AR, which has been widely used for the study of prostate cancer. We are currently investigating the effect of IL-6 on androgen responsiveness in androgen-sensitive human prostate cancer cells

using antibody against phospho-specific p44/42 ERK1/2 and reprobred with total p44/42 ERK1/2 or antibody against phospho-specific Akt (p-Akt Ser473) and reprobred with total Akt. The cells were cultured in RPMI 1640 with 10% FBS for 24 h and switched to either 10% FBS or 10% CS-FBS, and culture continued for another 72 h. Cell lysis were extracted and used for the assays.

expressing a wild-type AR. Nevertheless, this study provides the first experimental evidence that IL-6 induces the transition of prostate cancer from an androgen-dependent to an androgen-independent phenotype, which corresponds to the induction of PSA expression through activation of AR. The androgen-independent phenotype induced by IL-6 in LNCaP cells is accompanied by significant activation of the Stat3 and MAPK signal pathways.

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