

Interleukin-6 Undergoes Transition From Growth Inhibitor Associated With Neuroendocrine Differentiation to Stimulator Accompanied by Androgen Receptor Activation During LNCaP Prostate Cancer Cell Progression

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BACKGROUND. Interleukin-6 (IL-6) has been implicated in the modulation of growth and differentiation in many cancers, and is associated with poor prognosis in renal cell carcinoma, ovarian cancer, lymphoma, melanoma, and prostate cancer. The effects of IL-6 on the growth of LNCaP human prostate cancer cells are puzzling with some groups showing growth stimulation, while others showing growth inhibition. In this study, we investigated the discrepancy of the effects of IL-6 on prostate cancer cells.

METHODS. Series of lower and higher passages of LNCaP cell sublines were generated by a long-term exposure of LNCaP cells in IL-6-containing culture media. The characteristics of these cell sublines were analyzed and the potential roles of neuroendocrine (NE) differentiation and androgen receptor (AR) activation were examined.

RESULTS. We demonstrated that while short-term treatment of IL-6 inhibits LNCaP cell growth by a paracrine mechanism associated with NE differentiation, long-term treatment of IL-6 promotes LNCaP cell growth by an autocrine mechanism accompanied by an activation of AR signaling. In the lower passages (less than 28 passages) of LNCaP cells treated with IL-6, the cell growth was severely retarded which is associated with NE-like morphology and increased expression of NE markers such as neuron-specific enolase (NSE) and chromogranin A (ChgA), and loss of AR expression. However, in the higher passages (higher than 42 passages) of LNCaP cells treated with IL-6, cells started to express endogenous IL-6. At the same time, NE characteristics were disappeared, AR signaling was activated and cells growth was accelerated. Knocking down the AR activation of the higher passages of LNCaP cells abolished autocrine IL-6-induced growth stimulation.

CONCLUSIONS. These studies suggest that acquisition of endogenous IL-6 production after prolong exposure of prostate cancer cells to IL-6 may contribute to an autocrine cell growth stimulation. Furthermore, the transition of IL-6 from a paracrine growth inhibitor to an autocrine growth stimulator suggests that IL-6 plays an important role during prostate cancer progression, possibly androgen-independent progression. *Prostate* 67: 764–773, 2007.

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KEY WORDS: IL-6; neuroendocrine; androgen receptor; PSA; prostate cancer

INTRODUCTION

Interleukin-6 (IL-6) is a pleiotropic cytokine of M_r 21,000–28,000 that regulates the immune response, modulates normal and cancer cell growth, differentiation, and cell survival [1,2]. IL-6 binding to IL-6 receptors including gp80 (α chain) and a signal transducer, gp130 (β chain), initiates signaling through several major signaling pathways including the Janus

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kinase (JAK)-Signal Transducers and Activators of Transcription (STAT) pathway, mitogen-activated protein (MAP) kinase pathways, and the phosphatidylinositol (PI) 3-kinase-AKT pathways [2,3].

IL-6 is associated with poor prognosis in renal cell carcinoma, ovarian cancer, lymphoma, melanoma, and prostate cancer [4,5]. The expression of IL-6 and its receptor has been consistently demonstrated in human prostate cancer cell lines and clinical specimens of prostate cancer and benign prostate hyperplasia [6–8]. Multiple studies have demonstrated that IL-6 is elevated in the sera of patients with metastatic prostate cancer and that the levels of IL-6 correlate with tumor burden, serum PSA, and clinically evident metastases [9,10]. In addition, serum IL-6 levels are elevated in men with androgen-independent prostate cancer compared to normal controls, benign prostatic hyperplasia, prostatitis, and localized prostate cancer [9]. Collectively, these data suggest that elevated IL-6 levels are associated with the lethal phenotype of prostate cancer.

In addition to the clinical data that IL-6 is associated with androgen-independent prostate cancer, experimental studies demonstrate that IL-6 plays a critical role in prostate cancer cell growth and differentiation [11]. IL-6 modulates androgen receptor (AR)-mediated gene expression by regulating the AR activation in LNCaP cells [12–15]. Overexpression of IL-6 enhanced PSA mRNA expression in LNCaP cells and can partially rescue LNCaP cells from growth arrest induced by androgen deprivation therapy [16]. In addition, overexpression of IL-6 protects LNCaP cells from undergoing apoptosis induced by androgen deprivation therapy [17]. Okamoto et al. demonstrated that IL-6 functions as a paracrine growth factor for the human LNCaP androgen-sensitive prostate cancer cells and an autocrine growth factor for the human DU145 and PC3 androgen-insensitive prostate cancer cells [18]. In contrast to the role of IL-6 in promoting LNCaP cell growth, it has also been reported that IL-6 mediates LNCaP cell growth arrest and induces neuroendocrine (NE) differentiation [19–23].

NE cells are identified by the morphologic difference and the presence of neurosecretory granules and neuronal makers, such as chromogranin A (ChgA) and neuron-specific enolase (NSE) [24]. NE cells comprise a minor fraction of the total epithelial population but the proportion increases as tumor progresses and become androgen-independent stage [24,25]. NE tumor cells in prostate carcinoma may influence tumor proliferation of surrounding cells by secreting such neuropeptides in a paracrine fashion [26]. It is suggested that neuropeptides secreted by NE cells may act as growth signal or confer anti-apoptotic capabilities onto non-NE cells in close proximity to NE cells as well [27]. The effect of NE cells on surrounding

prostate cancer growth has been tested in several ways. When NE cells were co-cultured in vitro, the growth of the prostate cell lines has been retarded [22]. However, when NE mouse allograft was implanted to LNCaP tumors, there was an increase in AR expression and continued prostate cancer growth in the absence of androgens [28]. The molecular basis by which NE cells arise in prostatic tumor and accelerated growth of surrounding cells remains largely unknown.

In this study, a series of LNCaP sublines including lower passage (expose to IL-6 less than 28 passages) and higher passage (expose to IL-6 more than 42 passages) were developed by a long-term treatment of LNCaP cells with IL-6 in vitro. "Bifunctional" effects of IL-6 on LNCaP cell growth were observed. Initial paracrine signal of IL-6 exerts growth inhibition of LNCaP cells which is associated with NE differentiation. After long-term IL-6 treatment, IL-6 becomes autocrine signal that stimulates LNCaP cell growth accompanied by an activation of AR signaling.

METHODS

Cell Culture

LNCaP cells were grown in RPMI 1640 with 10% FBS and penicillin/streptomycin at 37°C in a humidified atmosphere of 5% carbon dioxide. LNCaP cells were passaged once weekly in medium containing FBS and 5 ng/ml of IL-6 (R&D Systems, Minneapolis, MN). The cells of each passage were frozen for further analysis. LNCaP cells cultured continuously in 5 ng/ml of IL-6 for less than 28 passages were called lower passages of LNCaP cells, while higher than 42 passages called higher passages of LNCaP cells. The IL-6-treated LNCaP cells were named LNCaP-IL-6+ and the numbers after P indicates passage number. LNCaP cells passaged at the same time without addition of IL-6 were used as controls (LNCaP-IL-6-).

Morphological Analysis

LNCaP and LNCaP-IL-6+ sublines were imaged by photomicroscopy using phase-contrast optics (Leica, Rijswijk, The Netherlands).

Growth Assay

LNCaP cells passaged at the same time in the absence of IL-6 were used as controls. Control LNCaP cells and several of different passages of LNCaP-IL-6+ subline cells were seeded into 24-well plated at a density of 10^4 cells/well in regular medium without exogenous IL-6. Cells were counted each day using Cell Counter (Coulter, Coulter Corporation, Miami, FL).

Separately, control LNCaP cells (1×10^4) were seeded into 24-well plated in RPMI 1640 containing 10% FBS and incubated with increasing doses of IL-6 (0–20 ng/ml). Cells were counted after 3 days of incubation. To examine whether IL-6 antibody will block the effect of IL-6 on LNCaP cells, 20 $\mu\text{g}/\text{ml}$ of anti-IL-6 antibody (Sigma, St. Louis, MO) was added to the culture media containing 5 ng/ml of IL-6. For control, 20 $\mu\text{g}/\text{ml}$ of antibody against IgG was added.

Northern Blot Analysis

Total RNA was extracted from cells with TRIzol reagent (Life Technologies, Rockville, MD). Twenty micrograms of each sample was electrophoresed on 1.2% denaturing agarose gels and transferred to a nylon membrane (MSI, Westborough, MA). An IL-6 cDNA was labeled with [α - ^{32}P]dCTP (3,000 Ci/mmol, ICN, Costa Mesa, CA) using the Ready-To-Go DNA Labeling Beads (Amersham Pharmacia Biotech, Piscataway, NJ). Hybridization was carried out during 3 hr at 65°C in Rapid-hyb buffer (Amersham). Membranes were washed for 15 min at 65°C in $2 \times \text{SSC}$, 0.1% SDS (twice), $0.5 \times \text{SSC}$, 0.1% SDS, and $0.1 \times \text{SSC}$, 0.1% SDS. Radioactivity in the membranes was analyzed with a Storm Phosphoimager System.

IL-6 ELISA

IL-6 in the supernatants of LNCaP-IL-6+ cells of different passages was determined by ELISA according to the manufacturer's instruction (eBioscience, San Diego, CA).

Total and Nuclear Lysate Preparation

Whole cell extracts were obtained using High salt buffer with freezing-thawing procedure as described previously [29]. For nuclear lysate preparation, cells were harvested, washed with PBS once, and resuspended in a hypotonic buffer (10 mM, HEPES-KOH (pH 7.9), 10 mM KCl, 0.1 mM EDTA, 0.4% NP40, 0.5 mM PMSF, 0.5 mM DTT, 1 mM NaV, 20 mM NaF, and 1 $\mu\text{g}/\text{ml}$ protease inhibitor) and incubated on ice for 20 min. Nuclei were precipitated by 10,000 rpm centrifugation at 4°C for 10 min. After washing once with hypotonic buffer, the nuclei were lysed in a high salt buffer (10 mM, HEPES-KOH (pH 7.9), 400 mM NaCl, 1 mM EDTA, 1 mM DTT, 0.5 mM PMSF, 1 mM NaV, 20 mM NaF, 20% glycerol, and 1 $\mu\text{g}/\text{ml}$ protease inhibitor) and incubated at 4°C for 30 min with vigorous shaking. The nuclear lysate was precleared by 12,000 rpm centrifugation at 4°C for 15 min. Protein concentration was determined using the Coomassie Plus protein assay kit (Pierce, Rockford, IL).

Electromobility Shift Assay

The nuclear extracts were prepared and electromobility shift assay was performed by incubating nuclear extracts (5–10 μg) with AR consensus binding motif 5'-GGTACAGGGTGTCT-3' (Santa Cruz Biotechnologies, CA) in incubation buffer containing 10 mM HEPES, pH 7.9, 80 mM NaCl, 10% glycerol, 1 mM DTT, 1 mM EDTA, 100 $\mu\text{g}/\text{ml}$ poly(dI-dC). The protein-DNA complexes were resolved on a 4.5% non-denaturing polyacrylamide gel at room temperature, and the results were autoradiographed using Storm 840 Imaging system (Molecular Dynamics, Sunnyvale, CA).

Transfection and Reporter Gene Assays

Cells (1×10^5) (LNCaP and LNCaP-IL-6+) were plated in 24-well plate. Cells were transiently transfected with ARE-luc using Tfx 20 (Promega, Madison, WI) according to the manufacturer's instruction. After 1 hr of transfection, the DNA: liposomes mixture was removed, and the cells were treated with phenol red-free medium containing 5% CS-FBS with 10 nM of R1881. Cell extracts were obtained after 24 hr and luciferase activity was assayed using the Luciferase Assay System (Promega). Protein concentrations in cell extracts were determined using the Coomassie Plus protein assay kit (Pierce). Luciferase activities were normalized using the protein concentration of the sample. All transfection experiments were performed in triplicate wells and repeated at least four times. The relative luciferase activity was averaged from at least four independent experiments each with triplicate wells.

Western Blot Analysis

Whole cell extracts or nuclear extracts obtained were resolved in 10 or 12% SDS-PAGE. Proteins were then transferred to nitrocellulose membrane. After blocking overnight at 4°C in 5% milk in PBS—0.1% Tween-20, membranes were incubated for 1 hr at room temperature with antibodies of AR (Santa Cruz), NSE and ChgA (Chemicon, Temecula, CA), anti-RNA Polymerase II (Pol II, Promega) in 1% milk in PBS with Tween-20. Following secondary antibody incubation, immunoreactive proteins were visualized with an enhanced chemiluminescence detection system (Amersham Pharmacia Biotech, Buckinghamshire, England)

Statistical Analysis

Student's *t*-test (two-tailed) was used to determine the significance between control and different passages of LNCaP cells treated with IL-6, and $P < 0.05$ was considered significant.

RESULTS

Exogenous IL-6 Is a Paracrine Growth Inhibitor for LNCaP Cells

The effects of IL-6 on the growth of LNCaP cells *in vitro* have attracted extensive attention with some studies showing growth inhibition [19–22,30], while others showing growth stimulation [12–15,18]. To understand IL-6 function in prostate cancer cells, we initially examined the paracrine effects of IL-6 on LNCaP cell growth. Early passage (passage #21) of LNCaP cells were treated with increasing doses of IL-6 and the cell numbers were counted. IL-6 inhibited LNCaP cell growth in a dose-dependent manner (Fig. 1A). Addition of IL-6 antibody almost completely

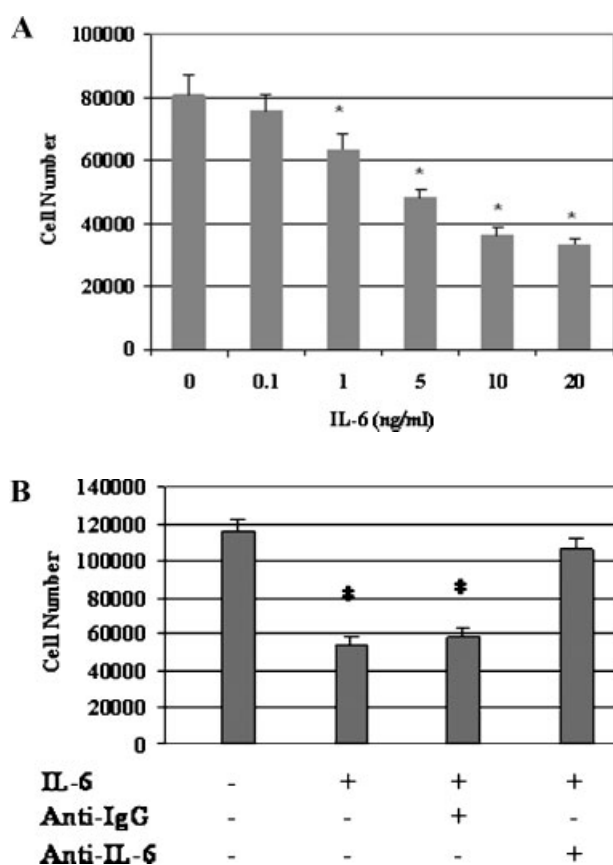


Fig. 1. Effects of exogenous IL-6 on growth of LNCaP cells. **A:** Dose-dependent inhibition of IL-6 on LNCaP cell growth. LNCaP cells (1×10^4) were seeded on 24-well plate in RPMI 1640 media containing 10% FBS. IL-6 was added at concentrations of 0–20 ng/ml. Cell number was determined after 3 days of incubation. **B:** Anti-IL-6 antibody reversed the effects of IL-6 on the growth of LNCaP cells. LNCaP cells were incubated with 5 ng/ml of IL-6 in the presence of 20 μ g/ml of antibody specific for IL-6 or 20 μ g/ml of control anti-IgG. The cell number was determined after 3 days incubation. Cell number was expressed as the mean \pm SD of triplicate samples. * indicates the significantly different ($P < 0.05$) from the control (0). Bars, SD.

abolished the growth inhibitory effect of IL-6 in LNCaP cells (Fig. 1B). These results together with other reports suggest that IL-6 inhibits LNCaP cell growth by a paracrine mechanism [19–22,30].

Endogenous IL-6 Functions as an Autocrine Growth Stimulator for LNCaP Cells

We have shown that IL-6 is a potential growth inhibitor for LNCaP cells when IL-6 was added exogenously to the cell culture. Since prostate cancer cells produce IL-6, we decided to examine the possible function of endogenous IL-6 in LNCaP cells. We ectopically expressed IL-6 by introduction of a full-length IL-6 cDNA into IL-6-negative LNCaP cells as described previously [14]. Two independent stable IL-6 transfectants (LN-S15 and LN-S17) were isolated that express high levels of IL-6 (2,465 and 2,743 pg/ml/ 10^6 cells, respectively) with a vector-alone control (neo). Endogenous overexpression of IL-6 by transfecting the IL-6 cDNA into LNCaP cells stimulated the cell growth (Fig. 2). These results suggest that IL-6 is a potential autocrine growth stimulator in LNCaP cells.

Long-Term IL-6 Treatment of LNCaP Cells Recapitulates the Bifunctional Effects of IL-6

Our results suggest that IL-6 exhibits “bifunctional” effects on LNCaP cells: a potential paracrine inhibitor and an autocrine stimulator for LNCaP cells. To understand the course of IL-6 transition from inhibitor to stimulator, LNCaP cells were treated with IL-6 at concentration of 5 ng/ml in the culture media

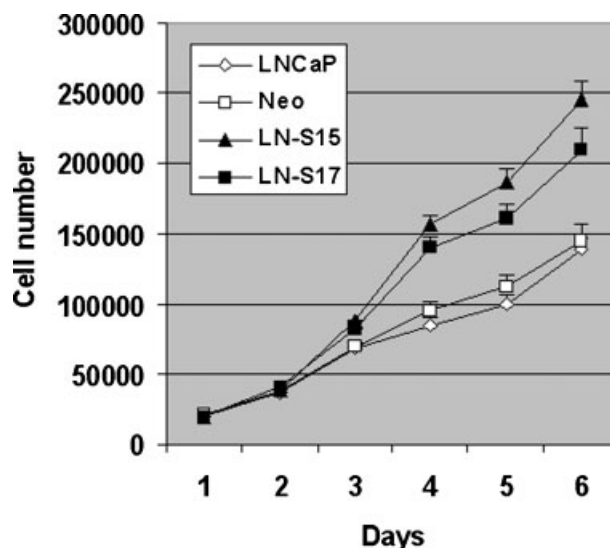


Fig. 2. Cell growth of the IL-6-expressing clones (LN-S15 and LN-S17) compared to the parental LNCaP and vector (Neo) control cells. Cells (1×10^4) were plated in triplicates in RPMI 1640 medium with 10% FBS. Cell number was counted by Coulter Counter. Bars, SD of triplicate samples.

continuously for a long term. Cells of every passage were frozen while generating those cells for further analysis. Series of passages including lower and higher passages of LNCaP cells treated with IL-6 were generated. The IL-6-treated LNCaP cells were named LNCaP-IL-6+ followed by passage number. The proliferation of cells of the lower and higher passages was analyzed in the absence of IL-6. LNCaP cells passaged at the same time in the absence of IL-6 were used as controls. Cells (LNCaP-IL-6+) of the lower passages less than P28 grow at much slower rate than control LNCaP cells, while cells of the higher passages (P42 and P58) treated with IL-6 proliferates rapidly (Fig. 3). Northern blot analysis showed that the control LNCaP and the lower passages (p10 and p28) of LNCaP cells treated with IL-6 express very low levels of IL-6 mRNA, if any. IL-6 mRNA express was elevated in the higher passages of LNCaP cells derived from long-term IL-6 treatment (Fig. 4A). Similarly, parental and the lower passages of LNCaP cells treated with IL-6 express very low levels of IL-6 protein in the supernatants, while the higher passages of LNCaP cells treated with IL-6 show much higher levels of IL-6 protein in the supernatants (Fig. 4B). These results are consistent with the report that LNCaP cells generated after long-term IL-6 treatment express IL-6 [31]. These results suggest that IL-6 functions as an inhibitor by a paracrine mechanism in the lower passages of LNCaP cells treated with IL-6, while a stimulator by an autocrine mechanism in the higher passages of LNCaP cells treated with IL-6.

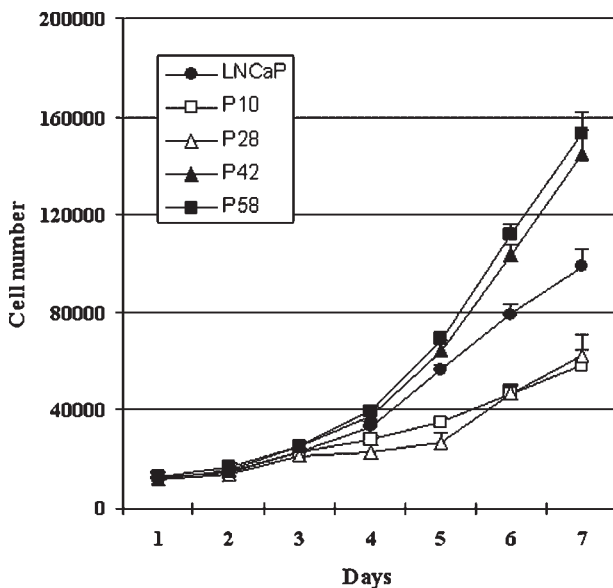


Fig. 3. Cell growth analysis. Cells (control LNCaP and different passages of LNCaP cells treated with IL-6 including P10, P28, P42, and P58) were plated in 24-well plates at a density of 1×10^4 . Cell number was determined by Coulter Counter. Three independent experiments were performed. Bars, SD of triplicate samples.

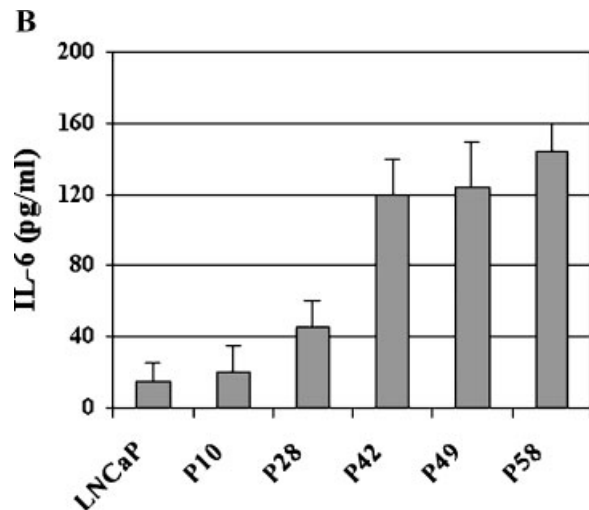
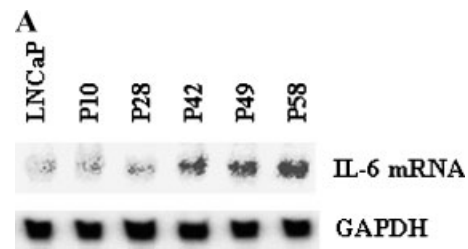


Fig. 4. IL-6 expression in control LNCaP cells and LNCaP cells treated with IL-6. **A:** IL-6 mRNA expression. Northern blot analysis was performed in control LNCaP cells, the lower passages of LNCaP cells treated with IL-6 (P10 and P28), and the higher passages of LNCaP cells treated with IL-6 (P42, P49, and P58). Northern blot analysis was performed using IL-6 probe. GAPDH is a control for equal loading. Each lane contains 20 μ g of total RNA. **B:** IL-6 protein secretion detected in culture supernatants. After 24 hr of incubation, 100 μ l of culture supernatant was taken and used for IL-6 ELISA analysis. The amount of IL-6 protein was normalized to cell number and represented as pg/ml/ 1×10^6 cells/24 hr. Values were expressed as the mean \pm SD of triplicate samples. Three independent experiments were performed.

NE Characteristics Were Developed by IL-6 Treatment

During the course of continuous treatment of LNCaP cells by IL-6, we observed significant morphological changes. Characteristics of NE cells were developed upon treatment with IL-6. The NE cells like morphology were detected as early as passage 2 and lasted until about passage 28 (Fig. 5A). Neuritic branching and extensions were observed and the growth was severely retarded. However, after passage 42, cells showed sudden acceleration in growth and the NE features were disappeared. The cells of higher passages of LNCaP cells treated with IL-6 show similar morphology as control LNCaP cells (Fig. 5A). The

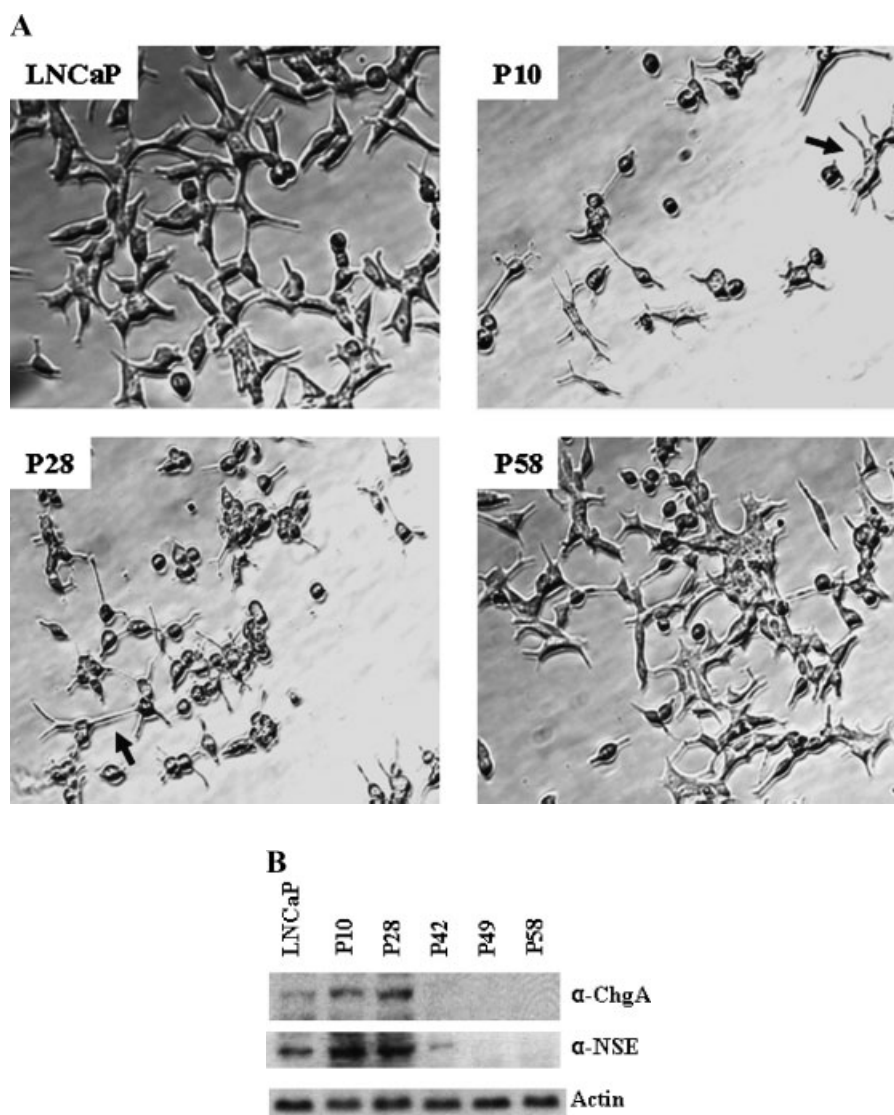


Fig. 5. Characteristics of neuroendocrine cell differentiation. **A:** Cell morphology. Cells of LNCaP and different passages of LNCaP-IL-6+ were grown in 60-mm³ dish in RPMI 1640 containing 10% FBS. Cell image was visualized using photomicroscopy. Arrows show neuritic branching and extension. **B:** Expression of NE markers. Cell extracts were isolated and 20 μ g of protein were subjected to Western blot analysis using antibodies against NSE and Chg A. Actin antibody was used as control.

expression of NE marker proteins, ChgA and NSE were determined using cell extracts of different passages. The levels of NSE and ChgA were increased in the lower passages of LNCaP cells treated with IL-6 compared to the control LNCaP cells, and decreased in the higher passages of LNCaP cells treated with IL-6 (Fig. 5B).

Activation of AR Signaling Pathway Accompanied by Growth Acceleration

Loss of AR expression has been associated with NE phenotype [32–34]. To determine the effect of long-term treatment of IL-6 on AR expression, the levels of

AR protein expression were determined by Western blot analysis in control LNCaP and the lower and higher passages of IL-6-treated LNCaP cells. The AR expression was lost in lower passages of LNCaP cells treated with IL-6 showing NE characteristics (Fig. 6A), consistent with other reports that loss of AR expression is associated with NE differentiation [32–34]. The AR is re-expressed in the higher passage of LNCaP cells treated with IL-6, of which the NE characteristics were disappeared (Fig. 6A). EMSA was performed using radiolabelled oligonucleotides of androgen responsive element (ARE) with nuclear extracts from the control LNCaP and the lower and higher passages of LNCaP cells treated with IL-6 to determine whether long-term

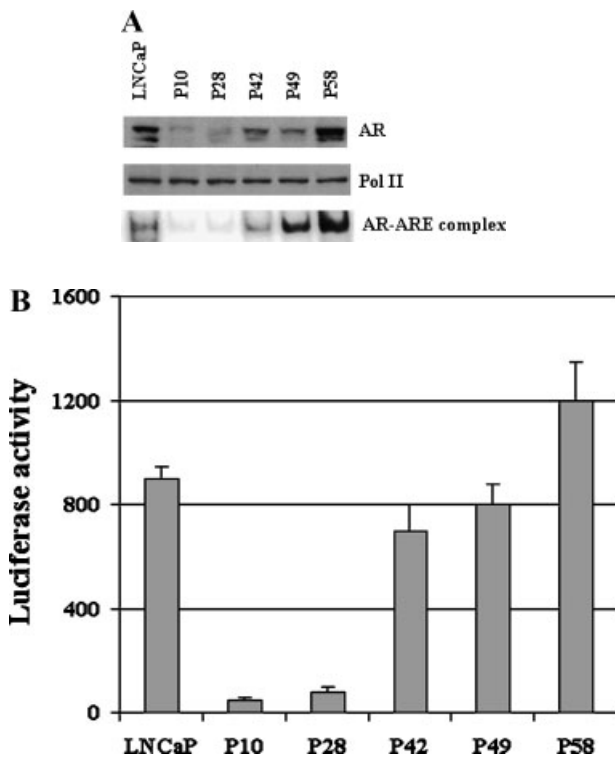


Fig. 6. Effects of continuous incubation of IL-6 on AR activation. **A:** AR activation. Nuclear AR expression in control LNCaP cells and in the lower and higher passages of LNCaP cells treated with IL-6. Nuclear extracts were subjected to Western blot analysis (20 μ g/lane) using an anti-human AR antibody. The expression of Pol II was used as marker for the integrity of the nuclear fractions. Bottom panel shows the formation of AR-ARE complexes. EMSA was performed using radiolabeled ARE oligonucleotides with whole cell extracts isolated from LNCaP and the lower and higher passages of LNCaP cells treated with IL-6. **B:** Transcriptional activity of ARE-luc. Cells of control LNCaP and different passages of LNCaP-IL-6 were transfected with ARE-luc and treated with 10 nM of DHT. Luciferase assay was performed after 24 hr incubation. Luciferase activity was normalized by protein concentration. Four independent experiments were performed.

IL-6 treatment affects DNA-binding activity of AR protein to ARE. The AR-ARE complex formation decreased in the lower passages of LNCaP cells treated with IL-6 compared to the control LNCaP and increased in the higher passages of LNCaP cells treated with IL-6 (Fig. 6A).

In an attempt to determine the effect of long-term IL-6 treatment on AR trans-activating activity, control LNCaP and the lower and higher passages of LNCaP cells treated with IL-6 were transiently transfected with an ARE-luciferase reporter plasmid, and luciferase activity was determined. The lower passages of LNCaP cells treated with IL-6 (LNCaP-IL-6+) showed lower luciferase activity than control LNCaP cells. The luciferase activity was increased in the higher passages of LNCaP cells treated with IL-6 (Fig. 6B).

Knock Down AR Expression Diminish IL-6 Autocrine Stimulation

Since IL-6 enhanced AR expression and activation in the fast proliferating higher passages of LNCaP cells treated with IL-6, we next tested whether knock down AR expression will affect the higher passages of LNCaP cell growth. The AR expression was knocked down by AR-specific siRNA (Fig. 7A), the growth of the higher passages of LNCaP cells treated with IL-6 was significantly reduced by AR siRNA compared to the control siRNA (Fig. 7B). These results suggest that growth stimulation of LNCaP cells by a long-term

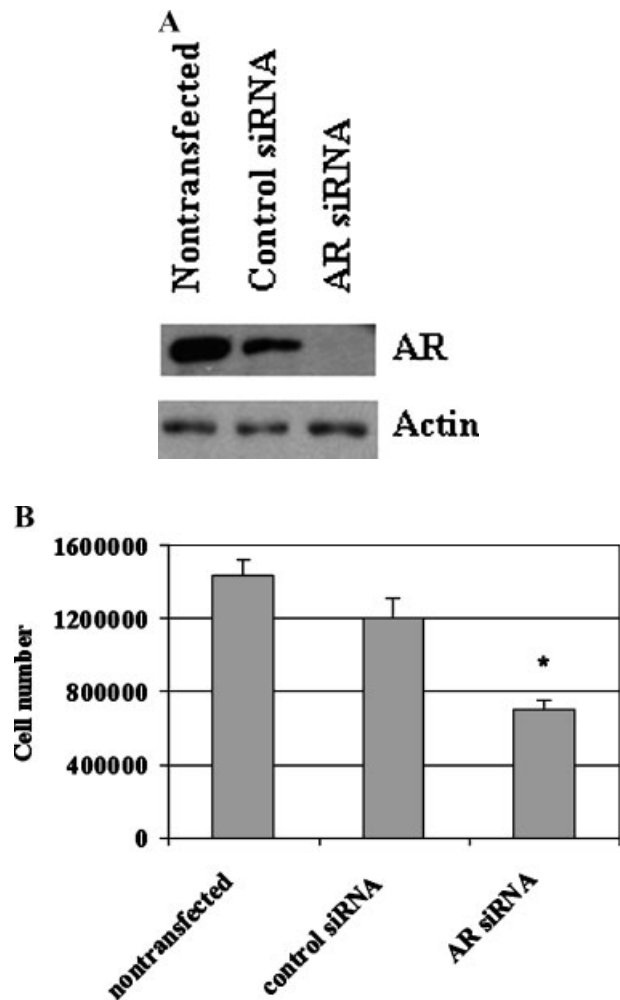


Fig. 7. Effects of knock down AR expression on IL-6-mediated LNCaP cell growth. The higher passage of LNCaP cells (P58) treated with IL-6 were transfected with either control siRNA or AR siRNA (5'-AAAGCAGCAGAAATGATTGCACT-3'). All transfections contained 2 μ g of total plasmid DNA. **A:** Whole cell protein extracts were isolated after 3 days cultured in the absence of IL-6 and subjected to Western blot analysis using anti-AR antibody. **B:** The cell number was counted following 3-day transfection. * indicates the significantly different ($P < 0.05$) from the control. Bars, SD.

treatment of IL-6 is mediated in part by activation of AR signaling.

DISCUSSION

In the present study, we intend to investigate the discrepancy of the role of IL-6 in prostate cancer cells. Using a serial of the lower and higher passages of LNCaP cell sublines generated by a long-term exposure of LNCaP cells in IL-6 containing culture media, we demonstrated that while short-term treatment of IL-6 inhibits LNCaP cell growth by a paracrine mechanism associated with neuroendocrine differentiation, long-term treatment of IL-6 promotes LNCaP cell growth by an autocrine mechanism accompanied by an activation of AR signaling.

Treatment of LNCaP cells with exogenous IL-6 showed a dose-dependent growth inhibition; such inhibition can be abolished by an addition of IL-6 antibody. These results are consistent with others that IL-6 is a growth inhibitor for LNCaP cells [20,30]. However, when LNCaP cells express endogenous IL-6 by introduction of a IL-6 cDNA in the cells, endogenously expressed IL-6 stimulates LNCaP cell growth, consisting with other published results showing that IL-6 stimulates LNCaP cell growth in vitro and in vivo [12–15,35]. In an attempt to clarify such contradictory role of IL-6 on LNCaP cell growth, we developed serial of the lower and higher passages of LNCaP cells in vitro by treating cells continuously with IL-6 (5 ng/ml) for prolonged period of time. When we analyzed cells of the lower and higher passages of LNCaP cells treated with IL-6, we found several interesting features: (1) Cell growth was severely retarded with initial treatment of IL-6 as a paracrine factor but accelerated with prolonged incubation when IL-6 becomes an autocrine factor; (2) Cells of early passages of LNCaP cells treated with IL-6 show morphology of NE-like cells but reverted to original morphology with prolonged treatment with IL-6; (3) AR signaling pathway was lost in cells of the lower passages treated with IL-6 but reappeared after prolonged IL-6 treatment; (4) Knock down the AR activation abolished autocrine IL-6-induced growth stimulation of LNCaP cells. The results may explain the conflicting results regarding the effects of IL-6 on growth of LNCaP cells. IL-6 exerts growth inhibitory effect by a paracrine mechanism, but functions as a stimulatory signal by an autocrine mechanism. It is noteworthy that the growth inhibitory effect of IL-6 was retained in the control LNCaP cells passaged in the absence of IL-6 in parallel to the higher passages of LNCaP cells generated in the presence of IL-6 (data not shown). Unlike the control LNCaP cells, in which the growth inhibitory effect of IL-6 by a paracrine mechanism can be abolished by an IL-6 antibody

(Fig. 1B), the growth stimulatory effects of IL-6 by autocrine mechanism in the higher passages of LNCaP cells treated with IL-6 can not be abolished by the IL-6 antibody (data not shown), suggesting that prolonged treatment of LNCaP cells with IL-6 retain irreversible growth advantages. We are currently investigating the molecular mechanisms underlying these changes.

It is shown that AR expression is decreased with emergence of NE cells induced by IL-6 treatment. Our data also showed downregulation of AR signaling pathway when NE cells are developed in the lower passages of LNCaP cells treated with IL-6. However, AR expression and its transactivation were significantly increased in the higher passages of LNCaP cells treated with IL-6. When AR expression was knocked down by means of siRNA, the growth of the higher passages of LNCaP cells treated with IL-6 was considerably decreased, suggesting that AR activation may contribute to the growth stimulation induced by the long-term IL-6 treatment. Our results that the AR expression in the higher passages of LNCaP cells treated with IL-6, however, appear different from the report that very high passages of LNCaP cells chronically treated with IL-6 lost AR expression [36].

Jin et al. [28] implanted NE-mouse allograft and showed increased AR expression and tumor growth and thus, they have suggested that NE component may produce factors that activate the expression and transactivation of AR. It is not clear yet how NE differentiation leads to acceleration in growth in prostate cancer cells. It has been suggested that neuropeptides secreted by NE cells stimulate the surrounding cells to grow [26,27]. We are currently testing whether neuropeptides are responsible for the sudden growth of the higher passages of LNCaP cells treated with IL-6.

The effects of IL-6 on the growth of LNCaP cells are puzzling with some groups showing growth stimulation, while others showing growth inhibition. Our observation that LNCaP cells cultured for an extended period of time acquired higher proliferation rates than their control cells passaged at the same time in the absence of IL-6 is in agreement with the report that LNCaP cells treated chronically with IL-6 have higher proliferation rates than their sister cells both in vitro and in vivo [31,37]. This study together with previous observations strongly suggest that IL-6 acts as a “bifunctional” cytokine for human prostate cancer cells during tumor progression: it acts as a growth inhibitor for LNCaP cells by a paracrine mechanism, whereas endogenously produced IL-6 stimulates LNCaP cell growth by an autocrine mechanism. This study also suggests that prolonged exposure of prostate cancer cells to IL-6 produced by surrounding cells may induce prostate cancer cells to acquire the ability of

endogenous production of IL-6. Acquisition of endogenous IL-6 production and its possible contribution to an autocrine cell growth stimulation may play an important role during prostate cancer progression, possibly androgen independent progression.

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