

Interleukin-4 Stimulates Androgen-Independent Growth in LNCaP Human Prostate Cancer Cells

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BACKGROUND. Clinical data showed that the levels of interleukin-4 (IL-4) are significantly elevated in serum of patients with ablation resistant prostate cancer. Previous studies demonstrated that IL-4 enhances androgen receptor (AR) activation mediated by NF- κ B in the absence or in the very low levels of androgen in prostate cancer cells. In this study, the role of IL-4 in promoting the growth of androgen-independent prostate cancer cells was examined.

METHODS. LNCaP cells were transfected with a full-length IL-4 cDNA and stable clones expressing IL-4 were selected. The growth of LNCaP cells expressing IL-4 was analyzed in vitro and in vivo both in the presence and absence of androgen.

RESULTS. Overexpression of IL-4 enhances the growth of androgen-sensitive LNCaP cells in culture media containing charcoal-stripped FBS condition (CS-FBC), and increases the sensitivity of LNCaP cells in response to androgen stimulation. The DHT-mediated cell growth could not be blocked by bicalutamide in IL-4 overexpressing LNCaP cells, but can be neutralized by bicalutamide in parental LNCaP and neo control cells. Furthermore, overexpression of IL-4 stimulates tumor growth of androgen-sensitive LNCaP cells both in intact and castrated male mice.

CONCLUSIONS. Overexpression of IL-4 increases the sensitivity of androgen-sensitive LNCaP prostate cancer cells in response to androgen stimulation and enhances the growth of LNCaP cells both in the presence and absence of androgen in vitro and in vivo. These studies suggest that IL-4 plays an important role in promoting androgen-independent prostate cancer growth. *Prostate* 68: 85–91, 2008. © 2007 Wiley-Liss, Inc.

KEY WORDS: IL-4; AR; prostate cancer

INTRODUCTION

Prostate cancer cells depend on androgen on its growth. Androgen regulates the expression of androgen-regulated genes such as prostate-specific antigen (PSA) through the binding of the androgen receptor (AR) to the androgen-responsive elements (AREs) in the promoters of the PSA. When androgen is depleted, prostate cancer cells initially undergo apoptosis and die. However, most patients will relapse to ablation resistant disease due to the growth of androgen recurrent cancer cells. Accumulating evidence demonstrates abnormal AR signaling contributes to androgen-independent growth of prostate cancer. AR can be activated by growth factors

and cytokines to display enhanced activity in the presence of low level of androgen or to function even in the absence of androgen [1–6].

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Interleukin-4 (IL-4) is a pleiotropic type I cytokine produced by T cells, mast cells, and basophils in response to receptor-mediated activation events [7]. IL-4 has biological effects on many immune cells, including B and T lymphocytes, mast cells, and macrophages, and plays a central role in regulating inflammatory and cell-mediated immune responses [8]. In addition to its effect on immune cells, IL-4 has a variety of other functions including effects on hematopoietic tissues, tissue adhesion, and inflammation [8]. IL-4 exerts its function through activation of the IL-4 receptor, designated IL-4R α , by tyrosine phosphorylation [9]. The receptor consists of a 140-kDa IL-4R α chain that binds IL-4 with high affinity (Kd 20 to 300 pM). IL-4R α activation results in tyrosine phosphorylation of multiple receptor-associated kinases including Janus-family (Jak) tyrosine kinases (Jak1, Jak2, and Jak3) [10–12], insulin receptor substrate (IRS-1/2) proteins [13,14], Shc [15], and signal transducers and activators of transcription (Stat6) [16] for the initiation of signal transduction. Among the molecules that interact with phosphorylated IRS-1/2 molecules are the regulatory subunit of phosphoinositide-3-kinase (PI3K) and the adapter molecule, Grb-2. These interactions lead to the activation of the PI3K and Ras/MAPK signaling pathways, respectively [8].

Clinical data showed that the levels of IL-4 are significantly elevated in serum of patients with ablation resistant prostate cancer [17,18]. Previous studies demonstrated that IL-4 activates AR-mediated gene expression by activation of the AR in a ligand-independent manner or in the presence of very low levels of androgen [19]. Further studies showed that IL-4-mediated AR activation involved in activation of NF- κ B [20]. The fact that IL-4 activates AR and NF- κ B suggest that IL-4 may involve in androgen-independent growth. Currently, the role of IL-4 in prostate cancer cell growth, particularly, in enhancing androgen-independent growth is unknown. In this study, we test the hypothesis that IL-4 expression stimulates androgen-independent growth of human prostate cancer cells.

METHODS

Cell Culture

LNCaP cells (passage number between 18 and 25) were grown in RPMI 1640 with 10% FBS and penicillin/streptomycin at 37°C in a humidified atmosphere of 5% carbon dioxide.

Transfection and Selection of Stable Clones Expressing IL-4 in LNCaP Cells

A cDNA encoding a full open reading frame of the human IL-4 was obtained from ATCC (Manassas, VA)

and co-transfected with pCDNA3.1-neo into LNCaP cells (passage number 18) using Tfx 20 (Promega, Madison, WI) according to the manufacturer's instruction. The stable clones were selected in the presence of G418, subcloned, and tested for their expression of IL-4 by ELISA analyses.

In Vitro Growth Assay

LNCaP, neo, and IL-4 overexpressing LNCaP cells were seeded into 12-well plated at a density of 5×10^4 cells/well in RPMI 1640 media containing either 10% FBS or 10% charcoal-stripped FBS. Cells were counted each day using Cell Counter (Coulter, Coulter Corporation, Miami, FL). For testing the effects of IL-4 on androgen responsiveness, cells (5×10^4) were seeded into 12-well plated in RPMI 1640 media containing 10% charcoal-stripped FBS with various concentrations of DHT in the presence and absence of bicalutamide. At the end of 3 days of incubation, cell numbers were counted.

In Vivo Growth Assay

Parental LNCaP, neo, and IL-4 overexpressing LNCaP cells (2×10^6 cells/site) were co-inoculated with matrigel (1:1) subcutaneously into the flank of male nude mice. The mice were monitored and the tumor volume was measured twice a week. When tumor volume reached about 300 mm³, mice were divided into two groups, one group was castrated and the other remained intact. The mice were monitored and tumor was measured twice a week.

ELISA Assay

Levels of IL-4 in the culture medium were determined by ELISA, with the use of polyclonal rabbit anti-human IL-4 as a solid-phase antibody and monoclonal mouse anti-human IL-4 as a second antibody, as described by the manufacturer's protocol (R & D Systems, Minneapolis, MN). IL-4 was expressed as a secretion rate into the culture medium in the units of pg/ml/24 hr/ 10^6 cells. The levels of PSA in the culture medium and serum of the tumor-bearing mouse were determined by ELISA as described previously [21,22].

Nuclear Lysate Preparation and Western Blot Analysis

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 μ g/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 μ g/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). Western blot analysis was performed as described previously [23]. Briefly, 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 and Pol II (Santa Cruz, CA) in 1% milk in PBS-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 transfected groups, and $P < 0.05$ was considered significant.

RESULTS

Effects of IL-4 on the Growth of LNCaP Cells In Vitro

We have demonstrated previously that IL-4 activates AR signaling in a low level of androgen or in the absence of androgen [19]. To examine the effects of IL-4 expression on prostate cancer cell growth, we developed IL-4 overexpressing cells by introduction of a full-length IL-4 cDNA into IL-4-negative LNCaP cells. Several stable transfectants containing IL-4 cDNA in the sense orientation and vector-alone controls were selected in the presence of G418, subcloned, and tested for their expression of IL-4 by ELISAs. Two stable IL-4 transfectants (C4A and C6A) expressing high levels of IL-4 (3,815 and 4,187 pg/ml/ 10^6 cells, respectively) were selected for additional studies.

LNCaP cells are androgen-sensitive cells. The cell growth is reduced when LNCaP cells are cultured in androgen-deprived condition in the charcoal-stripped FBS compared to normal FBS [21]. To examine whether IL-4 enhances LNCaP cell growth in the charcoal-stripped FBS condition (CS-FBS), parental LNCaP, neo control, and IL-4 overexpressing clones were cultured in media containing either FBS or CS-FBS, and the cell growth was determined. When the cells were grown in normal FBS media, IL-4 overexpressing cells, C4A and C6A, showed higher proliferation rate than its neo and

parental LNCaP cells (Fig. 1A). More interestingly, the IL-4 overexpressing LNCaP cells survived well in androgen-deprived condition compared to the parental LNCaP and neo control cells (Fig. 1B). To examine whether the effects of IL-4 were not LNCaP cell specific, we generated IL-4 overexpressing clones by transfecting IL-4 cDNA into AR-positive CWR22Rv1 prostate cancer cells [24,25]. Two stable IL-4 overexpressing clones (C2A and C8B) expressing high levels of IL-4 (7,055 and 4,580 pg/ml/ 10^6 cells, respectively) were selected. Similar phenomenon was observed in IL-4 expressing CWR22Rv1 clones (Fig. 1C,D). These results suggest that IL-4 expression enhances androgen-sensitive LNCaP cells survival in androgen-deprived condition in vitro.

Effects of IL-4 on Androgen Responsiveness in LNCaP Cells

LNCaP cells respond to androgen in a biphasic manner with a maximum stimulation at 1 nM of DHT and subsequent decrease at 10 nM of DHT [21]. To examine whether IL-4 expression affect the sensitivity of LNCaP cells in response to DHT treatment, IL-4 expressing LNCaP cells (C4A and C6A), parental LNCaP, and neo control cells were cultured in media containing charcoal-stripped FBS in the presence of different concentrations of DHT (from 0 to 10 nM). As expected, parental LNCaP and neo control cells respond to androgen in a biphasic manner with little growth stimulation between 0 and 0.01 nM of DHT, reached a maximum stimulation at 1 nM of DHT, and decreased at 10 nM of DHT (Fig. 2A). Addition of bicalutamide, blocked DHT-mediated LNCaP cell growth (Fig. 2B). Similar to the parental LNCaP and neo control cells, IL-4 expressing LNCaP cells (C4A and C6A) respond to androgen in a biphasic manner (Fig. 2A). However, DHT stimulated IL-4 expression LNCaP cell growth starting at concentration of 0.001 nM, reached maximum stimulation at 0.1 nM, and decreased at 1 nM of DHT (Fig. 2A). Interestingly, addition of bicalutamide was not able to block DHT-mediated cell growth in IL-4 overexpressing LNCaP cells (Fig. 2B). These results suggest that expression of IL-4 increases androgen sensitivity of androgen-sensitive LNCaP cells which cannot be blocked by anti-androgen treatment.

Effects of IL-4 on LNCaP Cell Growth In Vivo

Having demonstrated that IL-4 enhances the growth of androgen-sensitive LNCaP cells in the absence of androgen or in the presence of very low levels of androgen in vitro, we further examined the effect of IL-4 on the growth of androgen-sensitive LNCaP cells in vivo. IL-4 expressing LNCaP cells, parental LNCaP,

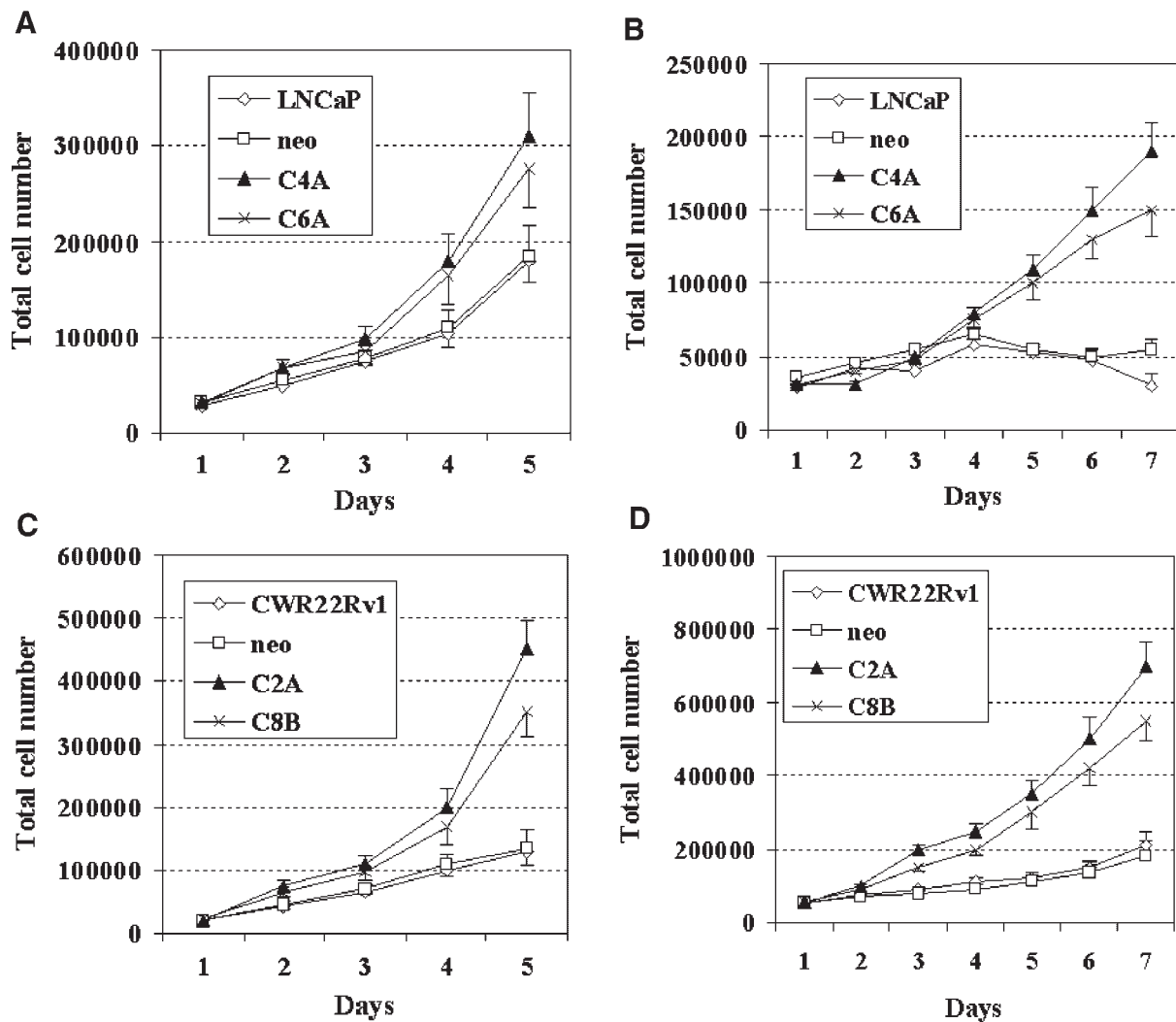


Fig. 1. In vitro cell growth. **A,B:** IL-4 overexpressing LNCaP cells (C4A and C6A), LNCaP, and neo cells. A: Cell growth in FBS condition; (B) Cell growth in CS-FBC. **C,D:** IL-4 overexpressing CWR22Rv1 cells (C2A and C8B), CWR22Rv1, and neo control cells. C: FBS condition; (D) CS-FBC. Cells (5×10^4) were plated into 12-well plate in RPMI 1640 media containing either 10% FBS or 10% CS-FBC. Cell number was determined by Coulter Counter. Each point represents at least four independent experiments.

and neo control cells were injected into nude mice subcutaneously and tumor growth was monitored twice a week. LNCaP cells expressing IL-4 developed tumors with high incidence (higher than 90%) and grew aggressively while LNCaP and neo control cells developed tumors with low incidence (less than 10%) and very slow growing tumors (Fig. 3). To test whether IL-4 enhances androgen-sensitive LNCaP cells grow tumor in the absence of androgen, the tumor-bearing mice from IL-4 expressing LNCaP cells were divided into two groups, one group was castrated and the other group remained intact. After castration, the tumor volume was measured twice a week. As shown in Figure 3, castration did not result in a significant decrease in tumor volume compared to the intact mice, suggesting that overexpression of IL-4 enhances

androgen-sensitive LNCaP cells grow in castrated male mice.

AR Activation is Enhanced in IL-4 Expressing LNCaP Cells

Previous studies demonstrated that IL-4 enhances AR activation in prostate cancer cells [19]. To test whether overexpression of IL-4 enhances the expression of an endogenous, androgen-regulated PSA, the expression of PSA was compared among IL-4 expressing LNCaP cells, parental LNCaP and neo control cells in the presence and absence of androgen. The levels of PSA protein secretion were comparable among the IL-4 overexpressing LNCaP cells and the parental and vector control LNCaP cells in the presence of androgen

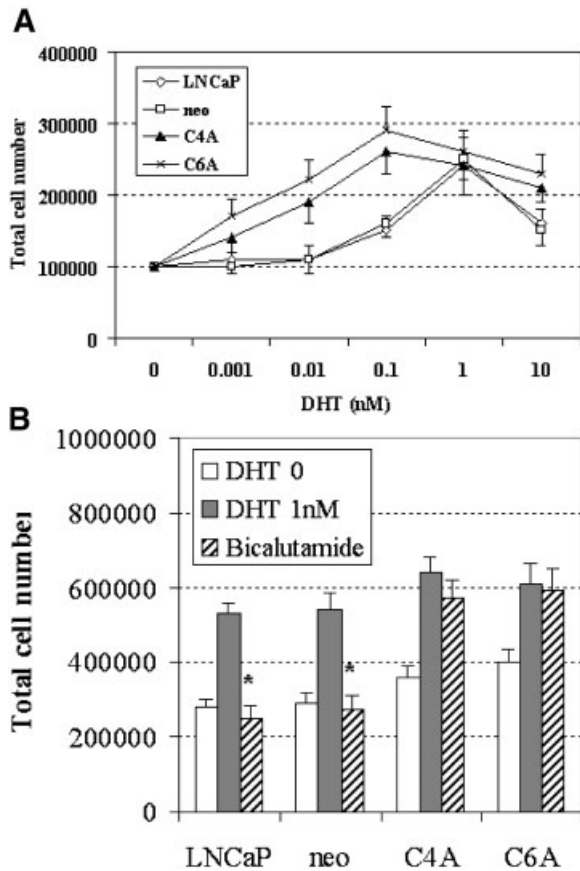


Fig. 2. Effects of DHT on cell growth. **A:** Effects of DHT on cell growth. IL-4 overexpressing cells (C4A and C6A), LNCaP, and neo control cells (5×10^4) were plated into 12-well plate in phenol red-free RPMI 1640 media containing 10% charcoal-stripped FBS with various concentrations of DHT as indicated. Cell number was determined by Coulter Counter. Each point represents three independent experiments. **B:** Effects of anti-androgen on DHT-mediated cell growth. IL-4 overexpressing LNCaP cells (C4A and C6A), LNCaP, and neo control cells (5×10^4) were plated into 12-well plate in phenol red-free RPMI 1640 media containing 10% charcoal-stripped FBS. The cells were treated with either 1 nM DHT or 1 nM DHT plus 10 μ M bicalutamide for 3 days. The cell number was determined by Coulter Counter. Each point represents at least three independent experiments. *, indicate statistical significance compared to 1 nM DHT treatment group.

(normal FBS condition). However, when the cells were cultured in phenol red-free medium supplemented with the charcoal-stripped FBS, in which the androgen was deprived, PSA protein secretion was decreased to almost undetectable level in the parental LNCaP and neo control cells (Fig. 4A). In contrast, PSA protein levels were significantly elevated in the IL-4 overexpressing LNCaP cells compared with the parental and vector control LNCaP cells (Fig. 4A), suggesting that overexpression of IL-4 can enhance endogenous PSA expression in the absence of androgen. In addition,

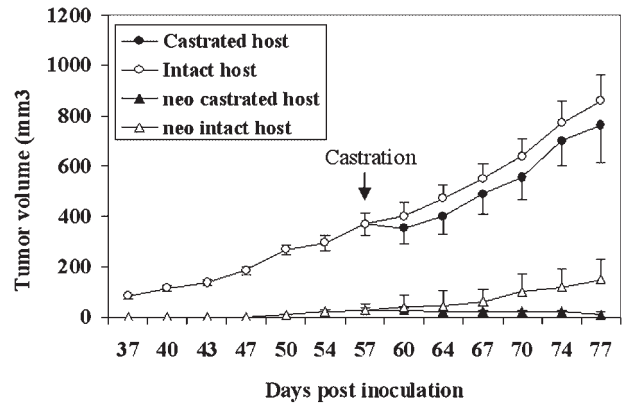


Fig. 3. Progression of LNCaP and IL-4 overexpressing LNCaP tumor xenografts in male athymic nude mice. IL-4 overexpressing LNCaP cells C4A, parental LNCaP, and neo control cells (2×10^6 cells/site) were subcutaneously co-inoculated with Matrigel (1:1) into the flank of male nude mice. Tumor volume was measured twice a week. When tumor volume reached about 300 mm³, the tumor-bearing mice were divided into two groups. One group was castrated, while another was left intact. The arrow indicated starting point of castration. Points, mean of tumor volumes; bars, SE.

tumors generated from IL-4 overexpressing LNCaP cells also produced high levels of circulating PSA in the serum of both intact male mice (8.6 ± 3.0 ng/ml/g of tumor) and the castrated male mice (9.4 ± 3.5 ng/ml/g of tumor).

The AR typically translocates to the nucleus to exert its function on gene expression. To examine whether overexpression of IL-4 affects the expression and translocation of AR, Western blot analysis was performed using nuclear extracts. As shown in Figure 4B, AR protein levels were comparable among the LNCaP cells overexpressing IL-4 and parental LNCaP and neo control cells when the cells were cultured in media containing FBS. However, the nuclear AR protein in parental LNCaP and neo control cells were decreased to almost undetectable level when the cells were cultured in CS-FBC. In contrast, the levels of nuclear AR protein were significantly elevated in LNCaP cells overexpressing IL-4 in CS-FBC (Fig. 4B). The increase in AR nuclear expression by overexpression of IL-4 is consistent with elevated PSA protein levels observed when the cells were cultured in CS-FBC (Fig. 4A).

DISCUSSION

IL-4 is significantly elevated in serum of patients with ablation resistant prostate cancer compared with values in hormone-sensitive prostate cancer, and the levels of IL-4 directly correlated with elevated PSA [17]. We previously demonstrated that IL-4 enhances PSA expression by activation of AR signaling in LNCaP

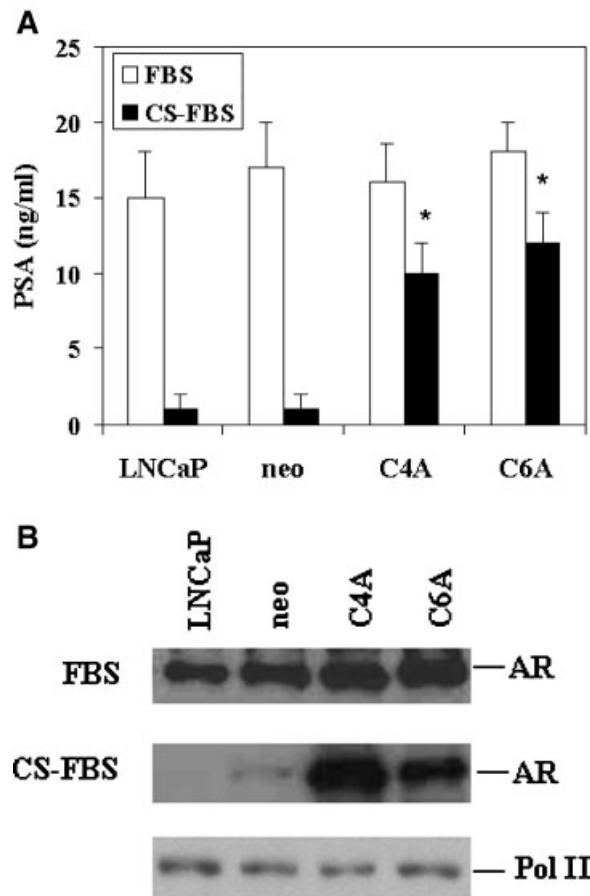


Fig. 4. Effects of IL-4 on AR activation. **A:** PSA protein secretion in culture supernatant. IL-4 overexpressing LNCaP cells, parental LNCaP, and neo control cells were grown in 100 mm culture dish in RPMI 1640 media containing either 10% FBS or 10% charcoal-stripped FBS for 3 days. The media was changed to fresh media and the culture supernatants were obtained after 24 hr and the levels of PSA proteins secretion were determined by PSA ELISA analysis. Bars, SE; *, indicate statistical significance compared to the levels in LNCaP, and neo control cells cultured in CS-FBS. **B:** AR protein expression. IL-4 overexpressing LNCaP cells, parental LNCaP and neo control cells were grown in 100 mm culture dish in RPMI 1640 media containing either 10% FBS or 10% charcoal-stripped FBS for 3 days. Nuclear protein was extracted and used for Western blot analysis using antibody specific against AR. The Pol II antibody was used as the loading control.

prostate cancer cells [19]. Furthermore, we demonstrated that NF- κ B activation by IL-4 plays critical role in mediating IL-4 induced AR activation and enhanced PSA expression in prostate cancer cells [20]. However, the effects of IL-4 on prostate cancer cell growth and androgen-independent progression are currently unknown. In this study, we investigated the role of IL-4 in prostate cancer cell growth and androgen-independent progression and demonstrated that IL-4 enhances androgen-sensitive LNCaP cell growth

in vitro and in vivo both in the presence and absence of androgen. These results suggest that overexpression of IL-4 promotes androgen-independent growth of androgen-sensitive LNCaP cells.

LNCaP cells are androgen-sensitive prostate cancer cells and their growth can be suppressed by androgen deprivation through culturing the cells in media containing charcoal-stripped FBS. Our data demonstrated that overexpression of IL-4 increases LNCaP cell growth in media containing either FBS or CS-FBS. However, the effects of overexpression IL-4 on the growth of LNCaP cells in media containing FBS are much less compared to the cells cultured in androgen-deprived CS-FBS as suggested by our data (Fig. 1A,B). Under androgen-deprived CS-FBS, the number of parental LNCaP and neo control cells remain essentially unchanged within 7 days culture. In contrast, IL-4 overexpressing LNCaP cells continued to grow during that period. It is demonstrated that androgen-sensitive LNCaP cells undergo apoptotic cell death upon androgen withdrawal [26,27]. The fact that IL-4 enhances the growth of androgen-sensitive LNCaP cells in androgen-deprived condition suggests that IL-4 protects LNCaP cells from apoptotic cell death induced by androgen deprivation.

It is interesting to note that overexpression IL-4 in LNCaP cells increased sensitivity of cells in response to androgen stimulation in culture. LNCaP cells respond to androgen in a biphasic manner with very little stimulation at 0.001–0.01 nM of DHT and reach to a maximum stimulation at 1 nM of DHT. However, IL-4 overexpressing LNCaP cells respond to a very low level (0.001 nM) of DHT and reached a maximum stimulation at 0.1 nM of DHT. Furthermore, bicalutamide was able to block androgen-mediated cell growth in LNCaP cells, but failed to block androgen-mediated cell growth in IL-4 overexpressing LNCaP cells. The increased sensitivity of LNCaP cells by overexpression of IL-4 in response to low levels of DHT may have important clinical implication in prostate cancer cell growth in androgen-deprived condition. Under androgen ablation condition, either by chemical or surgical castration, the level of residual androgens in patients is by no means absolutely androgen free. The level of residual androgens alone may not sufficient to induce appreciable cell growth. However, as showed by our data that IL-4 can stimulate LNCaP cell growth in a very low level of androgen (0.001–0.01 nM), androgen-sensitive prostate cancer cells may utilize IL-4 to grow and survive in low levels of residual androgens under androgen deprivation condition.

In conclusions, we demonstrated that overexpression of IL-4 increases the sensitivity of androgen-sensitive LNCaP prostate cancer cells in response to androgen stimulation and enhances the growth of

LNcaP cells both in the presence and absence of androgen in vitro and in vivo. Taken together with clinical observation that the serum levels of IL-4 is significantly elevated in patients with ablation resistant prostate cancer, these studies suggest that IL-4 plays an important role in promoting androgen-independent prostate cancer growth.

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