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Interleukin-4 enhances prostate-specific antigen expression by activation of the androgen receptor and Akt pathway

Soo Ok Lee[1], Wei Lou[1], Min Hou[1], Sergio A Onate[2] and Allen C Gao*[,1]

[1]*Department of Medicine and Pharmacology & Therapeutics, Roswell Park Cancer Institute, Buffalo, NY 14263, USA;* [2]*Department of Cell Biology and Physiology, University of Pittsburgh Medical Center, Pittsburgh, PA 15213, USA*

Androgen receptor (AR) plays an important role in the development and progression of prostate cancer upon the action of androgen through the binding of the androgen-responsive elements (AREs) on the target genes. Abnormal activation of the AR by nonandrogen has been implicated in the progression of androgen-independent prostate cancer. The levels of interleukin-4 (IL-4) are significantly elevated in sera of patients with hormone refractory prostate cancer. The potential role of IL-4 on the activation of AR was investigated in prostate cancer cells. IL-4 enhances AR-mediated prostate-specific antigen (PSA) expression and ARE-containing gene activity through activation of the AR in the androgen ablation condition in human prostate cancer cells. The AR can also be sensitized by IL-4 and activated by significantly lower levels of androgen (10 pM of R1881) in prostate cancer cells. IL-4 enhances nuclear translocation of AR and increases binding of the AR to the ARE in LNCaP prostate cancer cells. Blocking of the Akt pathway by an Akt-specific inhibitor LY294002 abrogates IL-4-induced PSA expression and AR signaling. These results demonstrate that IL-4 enhances PSA expression through activation of the AR and Akt signaling pathways in LNCaP prostate cancer cells. Understanding IL-4-induced signaling leading to abnormal activation of AR will provide insights into the molecular mechanisms of androgen-independent progression of prostate cancer cells.
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*Correspondence: AC Gao, Grace Cancer Drug Center, Department of Medicine and Pharmacology & Therapeutics, Roswell Park Cancer Institute, Elm & Carlton Streets, Buffalo, NY 14263, USA;
E-mail: allen.gao@roswellpark.org
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Introduction

Androgen plays an important role in the development and progression of prostate cancer (Isaacs, 1999). The growth of prostate epithelial cells requires a physiological level of androgen, both to stimulate proliferation and inhibit apoptotic death (Isaacs, 1994). Androgen binds to the androgen receptor (AR), which triggers interaction of AR to specific androgen-responsive elements (AREs) in the promoters of androgen-regulated genes such as prostate-specific antigen (PSA) (Trapman and Cleutjens, 1997). These interactions facilitate the activation or repression of genes regulating development, differentiation, and proliferation of target cells. Since prostate cancer initially occurs as an androgen-dependent tumor, almost all patients with advanced prostate cancer respond initially to androgen ablation therapy. However, virtually every patient will relapse due to the growth of androgen-independent cancer cells. Androgen-independent prostate cancer cells often continue to express the AR and androgen-responsive genes such as PSA (van der Kwast *et al*., 1991; Hobisch *et al*., 1995), suggesting that the AR becomes activated by an androgen-independent mechanism in androgen-independent prostate cancer cells. Although the molecular mechanisms underlying this androgen-independent growth are incompletely understood, several possible mechanisms have been suggested for this androgen-independent cancer growth including AR mutation or amplification (Barrack, 1996; Tilley *et al*., 1996; Linja *et al*., 2001) and recruitment of nonsteroid receptor signaling pathways such as cytokines and growth factors to activate AR in the absence of androgen (Culig *et al*., 1994; Hobisch *et al*., 1998; Craft *et al*., 1999; Yeh *et al*., 1999; Sadar, 1999; Chen *et al*., 2000; Wen *et al*., 2000; Lin *et al*., 2001; Ueda *et al*., 2001).

Interleukin-4 (IL-4) is a pleiotropic type I cytokine produced by T cells, mast cells, and basophils (Seder and Paul, 1994). 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 (Nelms *et al*., 1999). IL-4 acts not only as a major determinant for Th2 cell differentiation, but it also controls the specificity of immunoglobulin B cells switching to the expression of IgE and IgG (Vitetta

et al., 1985; Coffman *et al*., 1986; Gascan *et al*., 1991). In addition to the effect of immune cells, IL-4 has a variety of other functions including effects in hematopoietic tissues, tissue adhesion, and inflammation (Nelms *et al*., 1999).

IL-4 exerts its function through activation of the IL-4 receptor, designated IL-4Rα, by tyrosine phosphorylation (Smerz-Bertling and Duschl, 1995). IL-4Rα activation results in tyrosine phosphorylation of multiple receptor-associated kinases including Janus-family (Jak) tyrosine kinases (Jak1, Jak2, and Jak3) (Johnston *et al*., 1994; Witthuhn *et al*., 1994; Murata *et al*., 1996), insulin receptor substrate (IRS-1/2) proteins (Wang *et al*., 1993; Sun *et al*., 1995), Shc (Zhou *et al*., 1996), and signal transducers and activators of transcription (Stat6) (Shimoda *et al*., 1996) for the initiation of signal transduction. IRS proteins interact with SH2-containing signaling proteins, including the p85 regulatory subunit of phosphatidylinositol 3′-kinase (PI3 K), Grb-2, Shp-2, Nck, and PLCγ (Nelms *et al*., 1999).

Recent studies demonstrated that the levels of IL-4 are significantly elevated in hormone refractory prostate cancer compared with values in hormone-sensitive prostate cancer and that the levels of IL-4 directly correlated with elevated PSA (Wise *et al*., 2000). These observations suggest a potential role of IL-4 in the development and progression of androgen-independent prostate cancer. In this study, we examined the effect of IL-4 on PSA expression and activation of AR signaling in human prostate cancer cells. We demonstrate that IL-4 enhances PSA expression through activation of the AR and Akt signaling pathways in LNCaP prostate cancer cells.

Materials and methods

Cell culture

Human LNCaP prostate cancer cells were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA) and maintained in RPMI 1640 supplemented with 10% fetal bovine serum (FBS). CV-1 cells were routinely maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS. The cells were grown at 37°C in 5% CO_2 and 95% air. For IL-4 treatment, the cells were switched into 2% charcoal-stripped serum (Hyclone, CA, USA) 24 h prior to incubation and treated with different concentrations of IL-4 for variable times. The cells were also treated with either R1881 (NEN) alone or in combination of R1881 and hydroxyflutamide (Sigma) in the presence of 2% charcoal-stripped FBS.

Transfection and luciferase assay

At 24 h before transfection, 3×10^5 cells were plated in a six-well plate in phenol red-free medium containing 5% dextran-coated charcoal-stripped FBS (CS-FBS). Cells were transfected with a total amount of 2.5 μg of DNA using Superfect (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. The total amount of plasmid DNA used was normalized to 2.5 μg/well by the addition of empty plasmid. After 3 h, the DNA : liposomes mixture was removed, and cells were treated with phenol red-free medium containing 5% CS-FBS with either 10 nM R1881 (NEN) or in the absence of R1881. Cells were also treated with different concentrations of the recombinant IL-4 (R&D Systems, Minneapolis, MN, USA) or PD98059 (Promega, Madison, WI, USA), LY294002 (Promega, Madison, WI, USA), or AG490 (Sigma, St Louis, MO, USA). Cell extracts were obtained 36 h later and luciferase activity was assayed using the Luciferase Assay System (Promega, Madison, WI, USA). Protein concentration in cell extracts was determined by Coomasie Plus protein assay (Pierce, Rockford, IL, USA). Luciferase activities were normalized by protein concentrations of the samples. All transfection experiments were performed in triplicate wells and repeated at least four times.

Northern blot

Total RNA was extracted from cells with TRIzol reagent (Life Technologies, Rockville, MD, USA). RNA of 20 μg of each sample was electrophoresed in 1.2% denaturing agarose gels and transferred to a nylon membrane (MSI, Westborough, MA, USA). A 1.2-kb *Bam*HI fragment of the PSA cDNA was labeled with [α-^{32}P]dCTP (3000 Ci/mmol, ICN, Costa Mesa, CA, USA) using the Ready-To-Go DNA Labeling Beads (Amersham Pharmacia Biotech, Piscataway, NJ, USA). Hybridization was carried out during 3 h at 65°C in Rapid-hyb buffer (Amersham). Membranes were washed for 15 min at 65°C in $2 \times$ SSC, 0.1% SDS (twice), $0.5 \times$ SSC, 0.1% SDS, and $0.1 \times$ SSC, 0.1% SDS. Radioactivity in the membranes was analysed with a Molecular Imager FX System (Bio Rad, Hercules, CA, USA).

PSA protein analysis

PSA secretion was quantitated by ELISA with the use of anti-PSA as primary antibody as described by the manufacturer's protocol (Beckman Coulter, Fullerton, CA, USA). Equal numbers of cells were plated in phenol red-free RPMI containing 10% FBS. Cells were allowed to attach for 24 h, then the media were changed to phenol red-free medium supplemented with 2% charcoal-stripped serum and treated with different concentrations of IL-4 for variant time points. A volume of 50 μl of the supernatant was assayed for PSA.

Electromobility shift assay (EMSA)

Whole-cell extracts were prepared by using high-salt buffer (20 mM HEPES, pH 7.9, 20 mM NaF, 1 mM $Na_2P_2O_7$, 1 mM Na_3VO_4, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 0.5 mM PMSF, 420 mM NaCl, 20% glycerol, 1 μg/ml of leupeptin, and 1 μg/ml of 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 Plus protein assay kit (Pierce, IL, USA) according to the manufacturer's protocol. 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(dI-dC) by EMSA with radiolabled double-stranded AR consensus binding motif 5′-CTAGAAGTCTGGTA-CAGGGTGTTCTTTTTGCA-3′ (Santa Cruz Biotechnologies, CA, USA). The protein–DNA complexes were resolved on a 4.5% nondenaturing polyacrylamide gel containing 2.5% glycerol in $0.25 \times$ TBE 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 Molecular Imager FX System (Bio Rad, Hercules, CA, USA). For the supershift experiment, 20 μg of

cell extracts was incubated with monoclonal AR antibody (Santa Cruz Biotechnologies, CA, USA) for 1 h at 4°C prior to the incubation with a radiolabeled probe.

Nuclear lysate preparation

Nuclear protein extracts were prepared as described previously (Lin *et al*., 2001). Briefly, for nuclei preparation, cells were harvested, washed with PBS twice, and 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 determined by Coomassie Plus protein assay kit.

Western Blot

Whole-cell extracts were obtained by lysing the cells in RIPA buffer (1 × phosphate-buffered saline (PBS), 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, with freshly added protease inhibitors: 0.1 mM PMSF, 1 mM sodium orthovanadate, 30 μl/ml aprotinin). Whole-cell extracts were resolved in 12.5% 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 h at room temperature with anti-AR rabbit polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) or antiphosphorylated Akt (Cell Signaling Technologies, Beverly, MA, USA) diluted 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).

Results

IL-4 increases PSA mRNA and protein expression in LNCaP cells

PSA is a chymotrypsin-like serine protease and is synthesized primarily by normal, hyperplastic, and malignant prostate. The levels of PSA in serum correlate positively with the clinical stage of the disease, and the rebound of serum PSA is an indicator of hormone refractory prostate cancer (Wise *et al*., 2000). Since serum levels of IL-4 are elevated in hormone refractory prostate cancer compared to hormone-sensitive prostate cancer, we determined whether IL-4 will affect PSA expression in LNCaP human prostate cancer cells. LNCaP cells are androgen-sensitive cells and express endogenous PSA and mutant, but functional, AR. LNCaP cells were cultured in 10% FBS and the medium was switched into the androgen-deprived medium containing 2% charcoal-stripped serum for 24 h and the cells were exposed for 24 h to various concentrations of IL-4 (5–100 ng/ml). The levels of PSA mRNA were determined by Northern blot analysis. As shown in Figure 1a, the PSA mRNA was undetectable in LNCaP cells cultured in androgen-deprived charcoal-stripped serum. However, the levels of the PSA mRNA expression were upregulated upon IL-4 stimulation. An increase in the levels of PSA mRNA appeared to be dose dependent over the range of 5–50 ng/ml of IL-4, with a maximum increase in the levels of PSA mRNA achieved using a concentration of 50 ng/ml. The optimal

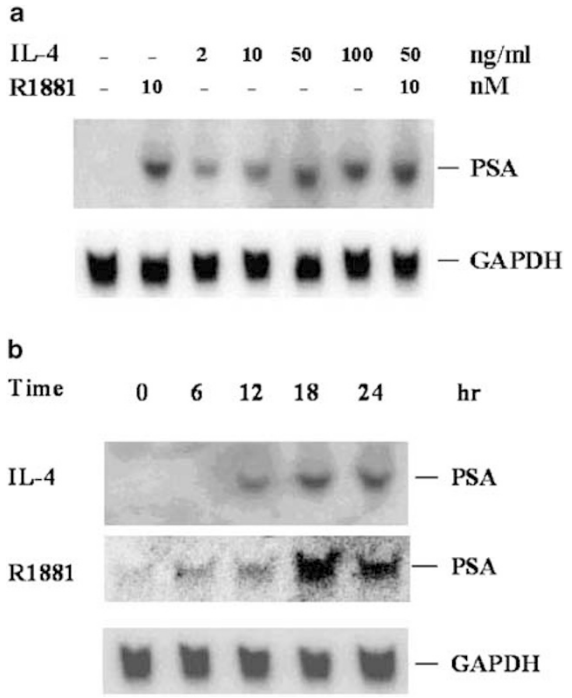

Figure 1 IL-4 increases PSA mRNA expression in LNCaP cells. (**a**) Northern blot analysis of PSA mRNA in LNCaP cells cultured in charcoal-stripped serum treated with either R1881 or various concentrations of IL-4 (2–100 ng/ml) for 18 h. (**b**). Time course of induction of PSA mRNA expression by IL-4 and R1881. Northern blot analysis of PSA mRNA in LNCaP cells cultured in charcoal-stripped serum condition treated with 50 μg/ml of IL-4 or 10 nM of R1881 in different time periods ranging from 0 to 24 h. GAPDH is a control for equal loading. Each lane contains 20 μg of total RNA

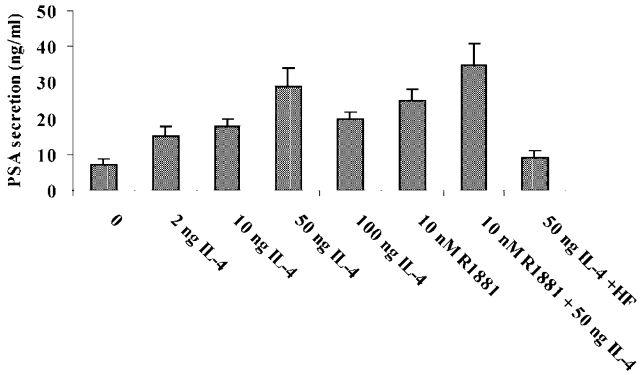

Figure 2 IL-4 increases PSA protein secretion. PSA secretion was quantitated by ELISA with the use of anti-PSA as primary antibody as described in the Materials and methods. Equal numbers of cells were plated in phenol red-free RPMI containing 10% FBS. Cells were allowed to attach for 24 h, then the media were changed to phenol red-free medium supplemented with 2% charcoal-stripped serum and treated with either 10 nM of R1881 or different concentrations of IL-4 (2–100 ng/ml), or with antiandrogen HF for 24 h. A volume of 50 μl of supernatant was assayed for PSA. Values were expressed as the mean ± s.d. of triplicate samples

induction of PSA mRNA by IL-4 in the concentration of 50 ng/ml was comparable with that achieved with 10 nM of R1881, a synthetic androgen. The optimal time for maximum induction of PSA mRNA expression in LNCaP cells was 18 h after the addition of IL-4, Figure 1b. The time frame related to PSA mRNA induction by IL-4 is similar to that in response to R1881, Figure 1b.

We then examined the effect of IL-4 on PSA protein secretion in LNCaP cells. As shown in Figure 2, IL-4 stimulates PSA expression in a dose-dependent manner, with a maximum increase in the levels of PSA secretion achieved using 50 ng/ml of IL-4. Similar to induction of PSA mRNA, the optimal induction of PSA secretion by IL-4 of 50 ng/ml was comparable with that achieved with 10 nM of R1881. Collectively, these results demonstrate that IL-4 enhances the AR-mediated PSA expression in the absence of androgen in LNCaP human prostate cancer cells.

IL-4 activates AR

PSA promoter is the best characterized androgen-responsive promoter and is typically regulated by androgen. Thus, we used PSA promoter as a model to examine the effects of IL-4 on AR signaling. To determine whether IL-4 activates AR signaling, the ability of IL-4 to activate the PSA promoter was determined. CV-1 cells were transiently transfected with an expression vector for the wild-type AR because these cells do not express the AR protein, and with the PSA promoter-luciferase reporter plasmid. This region of the PSA regulatory element contains the promoter and enhancer and has been demonstrated to be responsive to androgen stimulation (Pang *et al*., 1997). IL-4 increased PSAluciferase activity in a dose-dependent manner, with an optimal concentration of 50 ng/ml (data not shown). PSA promoter activity was increased eightfold by 10 nM of R1881 alone, threefold by IL-4, and 35-fold by a mixture of 10 nM of R1881 and 50 ng/ml of IL-4 (Figure 3a).

Androgen excites its function by binding to AR through AREs. We next examined whether AREs could be sufficient to be induced by IL-4. CV-1 cells were transiently transfected with an expression vector for the wild-type AR and with the AREs luciferase reporter plasmid. The reporter contains three repeats of the ARE region ligated in tandem with a luciferase reporter

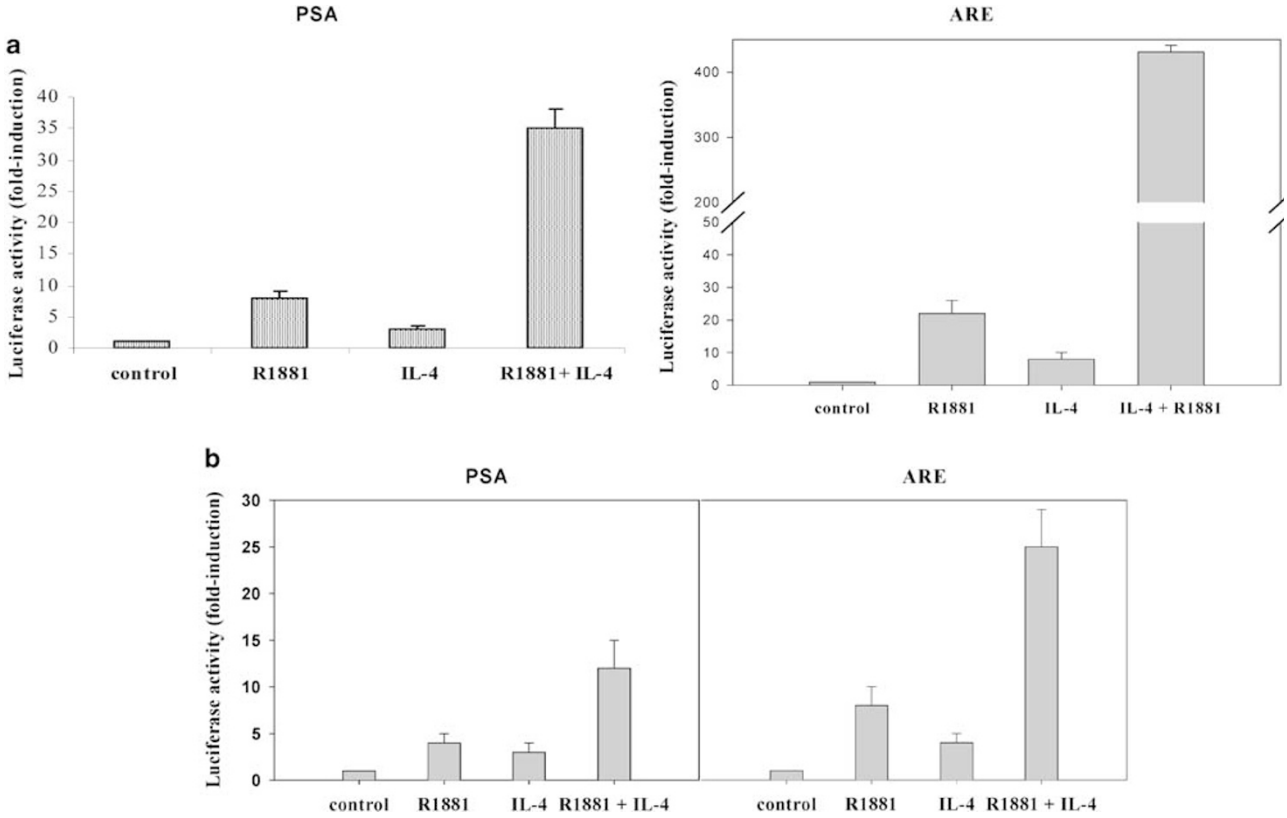

Figure 3 IL-4 enhances the activity of PSA and androgen-responsive promoter. Plasmids encoding PSA or ARE uciferase reporters were transfected into (**a**) CV-1 cells cotransfected with 0.5 μg of wild-type AR, (**b**) LNCaP cells. At 24 h before transfection, 3×10^5 cells were plated in a six-well plate in phenol red-free medium containing 5%CS-FBS. Cells were transfected with a total amount of 2.5 μg of DNA using Superfect. The total amount of plasmid DNA used was normalized to 2.5 μg/well by the addition of empty plasmid. After 3 h, the DNA : liposomes mixture was removed and the cells were treated with phenol red-free medium containing 5% CS-FBS either with 10 nM of R1881 or without R1881. Cells were also treated with 50 ng/ml of the recombinant IL-4. Cell extracts were obtained 36 h later and luciferase activity was assayed. Protein concentration in cell extracts was determined by Coomasie Plus protein assay. Luciferase activities were normalized by protein concentrations of the samples. All transfection experiments were performed in triplicate wells and repeated at least four times. Values were expressed as the mean $\pm$ s.d. of triplicate samples

(Onate *et al*., 1998). As shown in Figure 3a, the AREs luciferase activity was induced 22-fold by R1881, eightfold by IL-4, and 431-fold by the mixture of R1881 and 50 ng/ml of IL-4.

Since the CV-1 cells were not derived from prostate, we next investigated the effect of IL-4 on AR-mediated activation in LNCaP human prostate cancer cells. LNCaP cells express endogenous PSA and functional AR. As shown in Figure 3b, the PSA promoter activity was induced fourfold by R1881, threefold by IL-4, and 12-fold by the mixture of R1881 and 50 ng/ml of IL-4. The AREs luciferase activity was induced eightfold by R1881, fourfold by IL-4, and 25-fold by the mixture of R1881 and 50 ng/ml of IL-4.

IL-4 increases AR androgen sensitivity

The optimal concentration of R1881 for induction of PSA and AREs luciferase activity is 10 nM. R1881 alone in the concentrations of 0.01 and 0.1 nM does not induce PSA and AREs luciferase activity. To examine whether IL-4 increases AR androgen sensitivity, CV-1 cells were transiently transfected with an expression vector for the wild-type AR and with the AREs luciferase reporter. The cells were then treated with IL-4 in the presence of different concentrations of R1881 ranging from 0 to 100 nM. For the positive controls, all experiments were performed in parallel with R1881. As shown in Figure 4, R1881 in the concentrations between 0.01 and 0.1 nM failed to activate the ARE luciferase activity. However, low levels of IL-4 (0.1 ng/ml) increased the AREs luciferase activity by 55-fold in the presence of 0.01 nM of R1881, 109-fold in the presence of 0.1 nM of R1881, 172-fold in the presence of 1 nM of R1881, and 322-fold in the presence of 10 nM of R1881. Similar results that IL-4 significantly increased AR androgen sensitivity were obtained using 1 and 10 ng/ml of IL-4. These results demonstrate that IL-4 increases R1881 sensitivity at least by two orders.

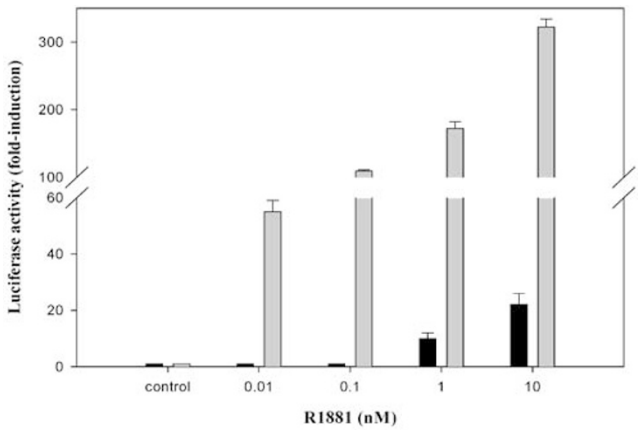

Figure 4 IL-4 modifies the sensitivity of AR for androgen. CV-1 cells were cotransfected with 0.5 μg of wild-type AR and 2 μg of ARE luciferase reporter. After 3 h, the DNA : liposomes mixture was removed and cells were treated with phenol red-free medium containing 5% CS-FBS with either R1881 ranging from 0 to 10 nM alone (black column) or R1881 (0–10 nM) plus 0.1 ng/ml of IL-4 (gray column). Cell extracts were obtained 36 h later and luciferase activity was assayed. Protein concentration in cell extracts was determined by Coomasie Plus protein assay. Luciferase activities were normalized by protein concentrations of the samples. All transfection experiments were performed in triplicate wells and repeated at least four times. Values were expressed as the mean ± s.d. of triplicate samples

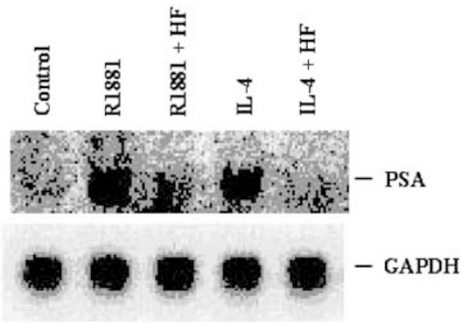

Figure 5 Hydroflutamide inhibits PSA mRNA induction by IL-4. LNCaP cells were cultured in charcoal-stripped serum and treated with either R1881 (10 nM), IL-4 (50 ng/ml), or combination with HF (10 μM) for 18 h. Cells were harvested and total RNA was isolated. Northern blot was performed using radiolabeled probes for PSA and GAPDH. Each lane contains 20 μg of total RNA

Flutamide blocks induction of AR activation by IL-4

To examine whether the induction of PSA expression and transfected androgen-responsive reporter constructs (PSA, AREs) by IL-4 requires functional AR, we performed experiments using hydroxyflutamide, an antiandrogen which is known to inhibit specifically the DNA-binding activity of the AR. Northern blot analysis showed that hydroxyflutamide blocked the induction of endogenous PSA mRNA (Figure 5) and PSA secretion (Figure 2) by IL-4 in LNCaP cells. As expected, hydroxyflutamide was able to block the induction of PSA mRNA expression induced by R1881. PSA luciferase activity induced by IL-4 was blocked (approximately 80%) by addition of hydroxyflutamide (data not shown). These results demonstrate that IL-4-induced PSA expression and luciferase activities of ARE-containing reporters can be blocked by antiandrogen hydroxyflutamide, suggesting that the functional AR is involved in IL-4 induction of androgen responsiveness.

IL-4 increases binding of the AR to the ARE

To test whether IL-4 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 cells treated with IL-4. LNCaP cells treated with IL-4 (50 ng/ml) for 30 min showed an increase in AR–ARE complex formation compared with the untreated LNCaP cells (Figure 6a). As a control, R1881 treatment (10 nM) enhanced AR–ARE complex formation. The specificity of this AR–ARE complex was demonstrated by supershift assay using an antibody specifically against AR (Figure 6b).

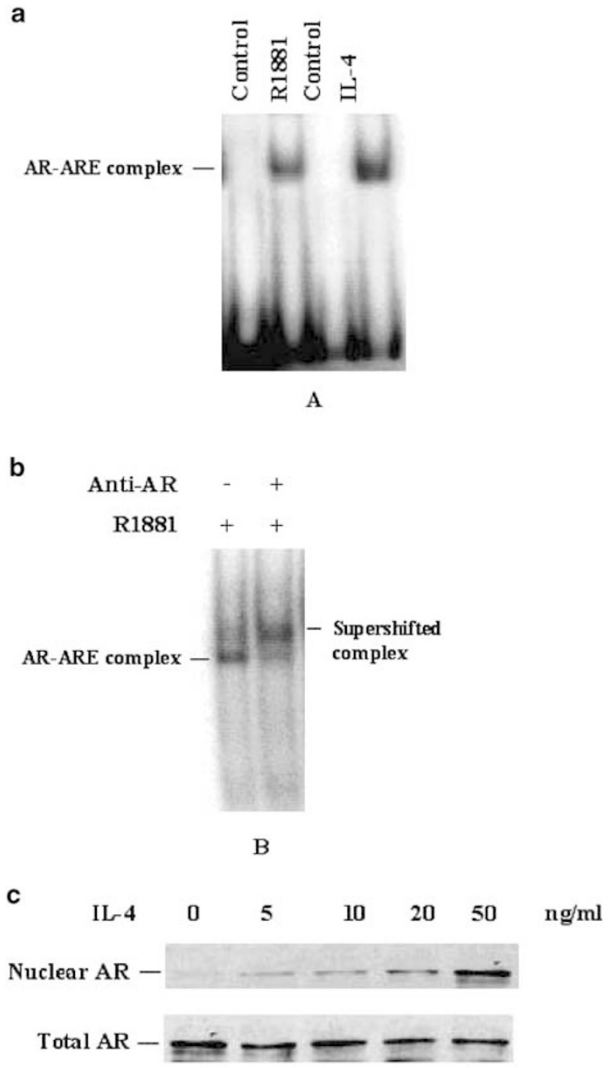

Figure 6 Effect of IL-4 on AR. (**a**) IL-4 enhances the formation of AR–ARE complexes. EMSA was performed using radiolabeled ARE oligonucleotides with whole-cell extracts isolated from LNCaP cultured in androgen-deprived CS-FBS media for 24 h with either R1881 (10 nM) or IL-4 (50 ng/ml) or control. (**b**) Supershift assay of LNCaP cell extracts obtained from R1881 (10 nM) treatment 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**) IL-4 enhances nuclear AR expression in LNCaP cells cultured in androgen-deprived charcoal-stripped serum. Total cellular extracts and nuclear extracts were subjected to Western blot analysis (40 μg/lane) using an antihuman AR antibody

Effect of IL-4 on whole cell and nuclear levels of AR protein

The AR typically translocates to the nucleus to exert its function on gene expression. To examine whether IL-4 affects the expression and translocation of AR, Western blot analysis was performed using cell extracts from either whole-cell extracts or nuclear extracts treated with IL-4. As shown in Figure 6c, IL-4 significantly enhanced the expression of AR in the nuclear compartment without alteration of the total AR expression (whole-cell extracts) in LNCaP cells cultured in the androgen-deprived charcoal-stripped serum.

Blocking the Akt pathway inhibits IL-4 induction of AR activation

We next examined the role of PI3K-Akt signaling pathway in mediating IL-4 in AR activation in prostate cancer cells. As shown in Figure 7, Akt was phosphorylated at Ser473 site in response to IL-4 stimulation in LNCaP cells. The Akt phosphorylation was increased in a dose-dependent manner by IL-4. However, the levels of total Akt protein expression were not altered by IL-4. LY294002, a PI3K-Akt specific inhibitor, completely abolished IL-4-induced PSA luciferase activity (Figure 8). AG490, a JAK-2 inhibitor (Meydan *et al*., 1996), also significantly inhibited IL-4-induced PSA-luciferase activity (Figure 8). Addition of PD98059, a MAPK inhibitor (Dudley *et al*., 1995), resulted in a minimal effect on PSA luciferase activity induced by the mixture of R1881 and IL-4. These results demonstrated that Akt was activated in response to IL-4

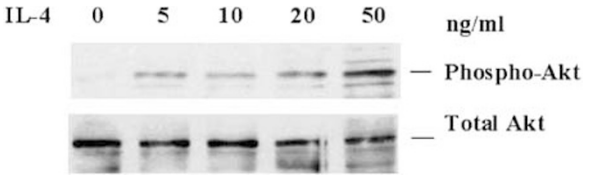

Figure 7 IL-4 activates Akt pathway. (**a**) The effect of IL-4 on Akt phosporylation in LNCaP cells. LNCaP cells were treated with an increasing dose of IL-4 ranging from 0 to 50 ng/ml in CS-FBS conditions for 18 h. Cell extracts were isolated and subjected to Western blot analysis using antibodies against phospho-specific Akt (p-Akt Ser473) and reprobed with total Akt

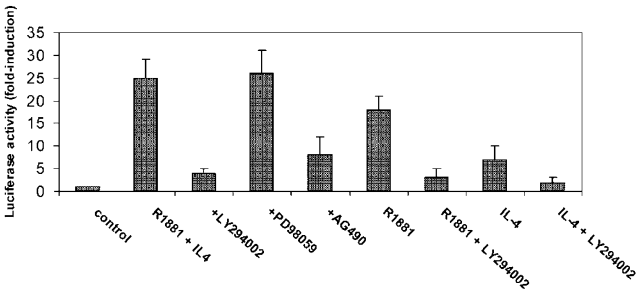

Figure 8 LY294002 inhibits PSA activation induced by IL-4. CV-1 cells were cotransfected with wild-type AR (0.5 μg) and PSA-luciferase reporter (2 μg). After 3 h, the DNA : liposomes mixture was removed and cells were treated with R1881 (10 nM) plus IL-4 (50 ng/ml) in phenol red-free medium containing 5% CS-FBS. The cells were also treated with R1881 (10 nM) with IL-4 (50 ng/ml) or R1881 (10 nM) and IL-4 (50 ng/ml) alone plus inhibitors such as LY294002 (20 μM) or PD98059 (20 μM), or AG490 (20 μM). Cell extracts were obtained 36 h later and luciferase activity was assayed. Protein concentration in cell extracts was determined by Coomasie Plus protein assay. Luciferase activities were normalized by protein concentrations of the samples. All transfection experiments were performed in triplicate wells and repeated at least four times. Values are expressed as the mean $\pm$ s.d. of triplicate samples.

stimulation, suggesting that PI3K-Akt is likely to be an important downstream signal in mediating IL-4 induction of AR activation in prostate cancer cells.

Discussion

The serum levels of IL-4 are significantly elevated in hormone refractory prostate cancer compared with values in hormone-sensitive prostate cancer, and the levels of IL-4 directly correlated with elevated PSA (Wise *et al*., 2000), suggesting that IL-4 may be involved in the development and progression of androgen-independent prostate cancer. However, the role of IL-4 in this androgen-independent progression is completely unknown. In this study, we demonstrated that IL-4 increases PSA mRNA and protein expression and increases androgen-responsive containing reporter transactivation through the activation of AR in prostate cancer cells. The AR can be sensitized by IL-4 and activated by significantly lower levels of androgen (10 pM of R1881). In addition, we demonstrated that PI3K-Akt is likely to be an important downstream signal in mediating IL-4 induction of AR activation in prostate cancer cells.

The expression of PSA is tightly regulated by androgen through the action of AR binding to AREs on the PSA promoter. However, PSA can also be regulated by other nonandrogenic factors. In this study, induction of PSA gene expression by IL-4 in human prostate cancer LNCaP cells has been demonstrated in mRNA level by both Northern blot and the PSA reporter gene constructs, and in protein secretion by ELISA. The induction of PSA mRNA and protein by IL-4 was shown to be dose dependent, with the optimal concentration at 50 ng/ml. High concentrations of IL-4 (100 ng/ml) decreased induction of PSA. The optimal induction of PSA expression by IL-4 in the concentration of 50 ng/ml was comparable with that achieved with 10 nM of R1881. The optimal time for maximum induction of PSA mRNA expression in LNCaP cells was 18 h after the addition of IL-4. The induction of PSA mRNA and protein expression by IL-4 in LNCaP cells were achieved in the androgen-deprived charcoal-stripped serum condition, suggesting that IL-4 can induce AR-mediated PSA expression in the androgen ablation condition or in the presence of very low levels of androgen. IL-4 can also induce PSA and AREs-containing reporter activity, consistent with the results that IL-4 induces PSA mRNA expression in LNCaP cells. The induction of PSA mRNA and protein expression and ARE-containing reporter activity by IL-4 were blocked by the nonsteroidal antiandrogen hydroxyflutamide. Hydroxyflutamide is thought to prevent the binding of androgen to AR. Thus, these studies suggest that IL-4 induction of PSA expression and ARE-containing gene reporter activity may act through an AR-dependent pathway. The antiandrogen property of hydroxyflutamide can be affected by the presence of certain cytokines. For example, in the presence of oncostatin M, hydroxyflutamide becomes androgen agonist (Godoy-Tundidor *et al*., 2002). The fact that hydroxyflutamide can block IL-4-induced AR activation suggests that hydroxyflutamide does not acquire agonistic properties in the presence of IL-4.

The involvement of AR in the induction of PSA gene expression by IL-4 was further demonstrated in LNCaP cells. EMSA experiments using radiolabeled oligonucleotides of the ARE with nuclear extracts from LNCaP cells treated with IL-4 showed an increase in the AR–ARE complex formation. The AR typically translocates to the nucleus to exert its function on gene expression upon binding to androgen. Western blot analysis demonstrated that IL-4 significantly enhanced the expression of AR in the nuclear compartment from LNCaP cells treated with IL-4. Taken together, these results demonstrate that IL-4 increases PSA expression through an AR-dependent pathway.

Androgen ablation is the most common and effective treatment for prostate cancer. However, low concentrations of androgen are present in sera of patients who receive hormone ablation therapy. Our results showed that IL-4 can enhance PSA mRNA and protein expression and activate ARE-containing gene in the absence of androgen. Additionally, IL-4 increases AR androgen sensitivity. IL-4 activates AR-mediated PSA and ARE-containing gene in a very low dose of androgen (10 pM of R1881). The concentration of androgen needed for maximal activation of the AR-mediated ARE-containing genes is reduced by a factor of 100 in the presence of very low levels of IL-4 (0.1 ng/ml). These results suggest that IL-4-activating AR may play an important role in the transition from androgen-dependent to androgen-independent prostate cancer after patients receive androgen ablation therapy.

We also investigated the role of signaling pathway such as PI3K-Akt in the induction of PSA expression by IL-4. We demonstrated that PI3K-Akt pathway is necessary for IL-4 induction of PSA expression using PI3 K inhibitor. IL-4 increases Akt activation in LNCaP cells. The induction of AR-mediated ARE-containing gene activity by IL-4 can be blocked by PI3 K inhibitor, but not by MAPK inhibitors. Thus, PI3K-Akt, but not MAPK, is likely to be an important downstream signaling molecule involved in the induction of AR-mediated ARE-containing gene activity by IL-4.

Cytokines have pleiotropic effects on cancer cell growth. To investigate the effect of IL-4 on human prostate cancer cell growth, LNCaP cells were cultured in the presence of different concentrations of recombinant IL-4 and cell numbers were determined. We found that although IL-4 enhances LNCaP cell growth *in vitro*, it failed to achieve statistical significance (data not shown).

In summary, the results of this study clearly show that IL-4 enhances PSA expression and ARE-containing gene activity by the activation of AR in human prostate cancer cells. The AR can be sensitized by IL-4 and activated by significantly lower levels of androgen (10 pM of R1881). In addition, we demonstrated that PI3K-Akt is likely to be an important downstream signal in mediating IL-4 induction of AR activation in

prostate cancer cells. Understanding the IL-4-induced signaling pathway leading to abnormal activation of AR and subsequent downstream AR-regulated genes will provide insights into the molecular mechanisms of androgen-independent progression of prostate cancer cells. Since IL-4 levels are elevated in the serum of hormone-refractory prostate cancer patients and that IL-4 can activate AR to increase PSA expression, it is likely that IL-4 may play a significant role in the progression of prostate cancer to androgen independence.

Abbreviations

IL-4, interleukin-4; AR, androgen receptor; ARE, androgen response element; PSA, prostate specific-antigen; Stat, signal transducers and activators of transcription; MAPK, mitogen-activated protein kinase; PI3 K, phosphatidylinositol (PI) 3-kinase; HF, hydroxyflutamide; CS-FBS, charcoal-stripped fetal bovine serum.

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