Interleukin-6 Regulates Prostate-specific Protein Expression in Prostate Carcinoma Cells by Activation of the Androgen Receptor

Alfred Hobisch, Iris E. Eder, Thomas Putz, Wolfgang Horninger, Georg Bartsch, Helmut Klocker, and Zoran Culig

Department of Urology, University of Innsbruck, A-6020 Innsbruck, Austria

ABSTRACT

Interleukin-6 (IL-6) levels are frequently elevated in sera of patients with metastatic prostate cancer. IL-6 receptors are expressed in prostate cancer cells lines, as well as in benign prostate hyperplasia and prostate cancer tissue specimens. The androgen receptor (AR) is a key transcription factor that is present in all stages of prostate carcinoma, even in therapy-refractory tumors. In an attempt to investigate possible cross-talk between IL-6 and androgen signal transduction cascades, we tested the effects of this cytokine on AR transcriptional activity. The regulation of AR activity by IL-6 was studied in DU-145 cells, which were cotransfected with the androgen-responsive reporter plasmid ARE2-TATA-CAT and the AR expression vector pSG5AR. We show that IL-6 up-regulates AR activity in a ligand-independent manner, as well as synergistically, with very low doses of the synthetic androgen methyltrienolone (5-10 pM). Therefore, AR activation by IL-6 may be operative in prostate cancer patients who have decreased androgen levels because of androgen ablation therapy. The maximal induction of reporter gene activity by IL-6 alone (50 ng/ml) was 67% of that stimulated by 1 nM of methyltrienolone. The nonsteroidal antiandrogen bicalutamide (Casodex) nearly completely inhibited AR activation by IL-6. IL-6 effects on AR activity were also abolished or greatly reduced by inhibitors of protein kinase A and C and mitogen-activated protein kinase pathways. In concordance with the results obtained in DU-145 cells, IL-6 induced AR-regulated prostate-specific antigen mRNA and protein in LNCaP cells. Stimulation of prostate-specific antigen protein secretion by IL-6 was antagonized by bicalutamide and inhibitors of protein kinase A and mitogen-activated protein kinase signaling pathways. Taken together, our data show for the first time that IL-6 is a nonsteroidal activator of the AR and that this activation is implicated in the regulation of prostate-specific proteins. Keeping in mind that IL-6, its receptor, and the AR are expressed in prostate cancers, cross-talk between IL-6 and AR signaling pathways may have clinical significance.

INTRODUCTION

IL-6 is a pleiotropic cytokine that regulates antigen-specific immune responses and inflammatory reactions (1). IL-6 is also implicated in modulation of growth and differentiation in many malignant tumors. Association of high serum IL-6 levels with poor outcome of the disease was observed in several solid and hematopoietic neoplasms (2, 3). The responsiveness of prostate cancers to endocrine therapy, which down-regulates the levels of circulating androgen and/or blocks the function of the AR, is only palliative, and nearly all carcinomas become therapy resistant. Two independent studies have demonstrated that IL-6 is elevated in sera of patients with metastatic prostatic carcinoma who failed androgen ablation therapy (4, 5).

IL-6 effects in target cells depend on the presence of its membrane receptor. The IL-6 receptor is expressed in tissues of epithelial and mesenchymal origin (6). It consists of the two subunits, the ligand-recognizing component (α-subunit) gp 80 and the signal-transducing component (β-subunit) gp 130 (7). Binding of IL-6 to the α-subunit leads to dimerization of the β-subunits and subsequent activation of Janus kinases. This step is followed by induction of tyrosine phosphorylation and nuclear translocation of signal transducers and activators of transcription factors and initiation of gene transcription (8).

IL-6 may act both as a positive and as a negative growth factor in target cells. It is one of the major growth-stimulatory factors for human myelomas (9). On the other hand, IL-6 is an inhibitor of early stage melanomas and mammary and lung carcinomas (10-12).

IL-6 receptors are expressed in the three human prostatic carcinoma cell lines, the androgen-insensitive DU-145 and PC-3 cell lines, and the androgen-responsive LNCaP cell line (13). Furthermore, it was shown that IL-6 receptor mRNA is present in ~80% of samples of benign prostate hyperplasia and in all prostate cancer samples (14). At present, it is not clear whether IL-6 acts as a stimulatory or as an inhibitory growth regulator in prostate carcinoma because the findings reported by various laboratories are discordant (13, 15-21).

AR protein is expressed in primary prostate carcinomas and in their metastases, including those obtained from patients who failed endocrine therapy (22-24). Several substances that bind to membrane receptors up-regulate the activity of the AR (25-27). It was demonstrated previously that IL-6 stimulates the activity of the human ER and glucocorticoid receptor (28, 29). Based on these findings, we hypothesized that IL-6 regulates the activity of the human AR in prostate cancer cells. We show in the present study that there is a cross-talk between the signal transduction cascade of IL-6 and the AR, which is relevant to modulation of cellular events in prostatic carcinoma.

MATERIALS AND METHODS

Cell Lines. Androgen-responsive LNCaP and androgen-insensitive DU-145 cells were obtained from the American Type Culture Collection and cultured at 37°C in an atmosphere of 5% CO2 in air. RPMI 1640 and MCDB 131 media were from HyClone (Logan, UT).

Chemicals. 3H-Labeled and unlabeled synthetic androgen methyltrienolone (R1881) and acetyl CoA were from New England Nuclear (Dreieichenhain, Germany). IL-6 was from R&D (Abington, United Kingdom). The stability of IL-6 was tested by the manufacturer in a bioassay after its placement at 37°C for 3 days. The values obtained from this sample were the same as those obtained from a properly stored protein. PKI was purchased from Sigma (Deisenhofen, Germany). The PKC inhibitor bisindoylmaleimide (GF 109203X) and the mitogen-activated protein kinase kinase inhibitor PD 98059 were provided by Calbiochem (La Jolla, CA). The nonsteroidal antiandrogen bicalutamide (Casodex) was kindly supplied by Zenea Pharmaceuticals (Macclesfield, United Kingdom). The Lipofectamine reagent was from Life Technologies, Inc. (Paisley, United Kingdom). The PSA Tandem-R immuno-radiometric assay was provided by Hybritech (San Diego, CA). The commercial MTT assay was purchased from Biomedica (Vienna, Austria).

Transfections. DU-145 cells (9 x 104/well) were seeded into 12-well plates 24 h before transfection and grown in RPMI with 10% FCS. Lipofectamine mediated transfection was performed in the absence of serum and antibiotics following the protocol that was described elsewhere (27). Briefly, the cells were cotransfected with the reporter plasmid ARE2-TATACAT and the AR
expression vector pSG5AR. In control experiments, the empty vector pSG5 was introduced into DU-145 cells instead of the AR expression vector. R1881, IL-6, the AR antagonist bicalutamide, and PK inhibitors were supplemented 20 h after transfection, and the cells were incubated in medium containing 2% charcoal-stripped FCS for the next 24 h. Alternatively, cotransfected DU-145 cells were incubated with IL-6 in serum-free medium. Afterward, the medium was removed, and the cells were frozen until the CAT assay was performed. The CAT assay procedure was described elsewhere (27). Subsequently, the ability of PKI to inhibit binding of the 3H-labeled R1881 to the AR was tested by a ligand binding assay that was performed as described by Culig et al. (27).

**Quantitative PCR for PSA Fragments.** Semiquantitative RT-PCR was performed as described previously by Eder et al. (30). Briefly, total RNA was isolated from cells using a guanidinium thiocyanate-acid phenol-chloroform (pH 4.0) extraction method. After denaturation (94°C for 2 min), 0.5 µg RNA was transcribed into cDNA using Ready To Go-Kit (Pharmacia, Uppsala, Sweden). cDNA was diluted in water to guarantee a quantitation of PCR fragments in the exponential phase of the reaction. One µl of diluted cDNA was amplified in a thermocycler (Biometra, Göttingen, Germany) with 50 s at 94°C, 1 min at 56°C, and 30 s at 73°C (28 cycles).

Primers were synthesized on a 381A DNA Synthesizer (Applied Biosystems, Vienna, Austria): PSA 418/21 sense, 5'-GGCACGTCCCTGTTGACCTCTC-3' (fluorescence-labeled) (Pharmacia); and PSA 939/21 antisense, 5'-CACCAGGAGCTTCCTTTTGCG-3'; β2-microglobulin was used as an internal control: sense, 5'-ATGCTCCGTGTTGAGACCATGT-3'; and antisense, 5'-AGAGCTACCTGTGGAGCAACCT-3' (fluorescence-labeled). For quantitation, PSA (1 µl) and β2-microglobulin (1 µl) samples were mixed in equal amounts with 2.5 µl of formamide. After denaturation at 95°C for 2 min, DNA fragments were separated electrophoretically on a 6% polyacrylamide gel by a 370A DNA sequencer (Applied Biosystems). Fluorescence was measured and analyzed with 672A Software 1.2 (Applied Biosystems). PSA mRNA levels were normalized against β2-microglobulin and presented as a PSA:β2-microglobulin ratio.

**PSA RIA.** The PSA protein was measured in cell culture supernatants from LNCaP cells. Cells (2 × 10⁵ cells/well) were grown in the presence of 10% FCS for 24 h in 24-well plates. Afterward, the cells were incubated in medium containing 2% charcoal-stripped FCS in the presence of androgen, IL-6, AR antagonists, and PK inhibitors for 72 h. In addition, PSA was measured in supernatants from cells incubated with IL-6 in serum-free medium. PSA values were expressed in relation to cellular protein levels, which were determined by the method described by Bradford (31).

**Cell Viability.** Cell viability after treatment of DU-145 and LNCaP cells with PK inhibitors was determined by means of the MTT assay. The assay was performed following experimental protocol described by Cronauer et al. (32).

**Statistical Analysis.** Mann-Whitney U tests were used to investigate significant differences among our experimental groups. Probability values of \( P < 0.05 \) were considered statistically significant.

**RESULTS**

**AR Activation by IL-6 in Transfected Prostate Cancer Cells.** Incubation of DU-145 cells, which were cotransfected with the androgen-inducible indicator gene ARE₂TATACAT and the AR expression vector pSG5AR, with increasing concentrations of R1881 yielded a dose-dependent increase in indicator gene activity. The maximal activity was achieved with 1 nM R1881. This androgen, at concentrations of 5 and 10 pm, caused 25 and 32% increase in reporter gene activity, respectively. IL-6 caused ligand-independent and ligand-dependent induction of CAT activity. Androgen-independent effects of IL-6 on AR activity were concentration dependent with maximal induction at 50 ng/ml (Fig. 1A). The mean value of reporter gene activity stimulated by 50 ng/ml of IL-6 was 67% of that induced by 1 nM R1881 (Fig. 1A). Similar stimulation of reporter gene activity by IL-6 was measured in experiments in which DU-145 cells were cultured in serum-free medium (data not shown). IL-6 did not induce reporter gene activity in experiments in which the AR expression vector was replaced with the empty vector pSG5. To obtain additional proof that IL-6 effects are AR-mediated, cotransfected DU-145 cells were coincubated with IL-6 and the antiandrogen bicalutamide. This compound was demonstrated previously to be able to block AR activity induced by PK activators (25-27). Bicalutamide was effective in inhibition of reporter gene activity not only in the presence of androgen but also in the presence of IL-6 (Fig. 1A). This finding confirms that IL-6 effects are mediated through the activation of the AR. Then we investigated the regulation of AR activity in DU-145 cells by low concentrations of androgen and IL-6 (Fig. 1A). A stronger synergistic effect was observed after cotreatment with R1881 and IL-6. In the presence of 10 ng/ml of IL-6, the concentration of R1881 needed for maximal activation of the AR was only 10 pm. Interestingly, synergistic effects of IL-6 were less pronounced when concentrations higher than 25 ng/ml were used. This type of activation of the AR was nearly completely blocked by bicalutamide as well (Fig. 1B).

**Down-Regulation of AR Activity by PK Inhibitors.** Recent studies suggested involvement of PK pathways in the regulation of transcriptional activity of the AR (25-27). We investigated the role of these signaling pathways in both steroidal and nonsteroidal activation of the AR. Initially, cotransfected DU-145 cells were incubated with androgen and either the PKA-specific inhibitor, PKI, the PKC inhibitor bisindolylmaleimide, or the mitogen-activated protein kinase kinase inhibitor PD 98059. These inhibitors did not affect the viability.
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of DU-145 cells, as determined by a MTT assay. PKI, at a concentration of 10 μg/ml, partially inhibited androgen-induced indicator gene activity. Fig. 2A shows that CAT activity measured in the presence of 1 nM R1881 is reduced by PKI by 41%. PKI itself does not inhibit binding of radiolabeled R1881 to the AR (data not shown). In contrast, substances that inhibit PKC and MAPK pathways do not down-regulate indicator gene activity induced by androgens. The effects of bisindolylmaleimide (1 μM) and PD 98059 (25 μM) on reporter gene activity in cells supplemented with IL-6 differ from those observed in the presence of androgen. Ligand-independent activation by IL-6 was blocked not solely by PKI but also by bisindolylmaleimide and PD 98059 (Fig. 2b). Furthermore, synergistic AR activation by androgen and IL-6 was also reversed by all three protein kinase inhibitors tested (data not shown). These experiments clearly demonstrated that PKA, PKC, and MAPK pathways are required for AR activation by IL-6.

Effects of IL-6 on PSA mRNA. We asked whether AR activation by IL-6 is relevant to the regulation of functional activity of an endogenous AR. The experiments were performed in LNCaP cells. The LNCaP AR, which contains a point mutation in the ligand-binding domain, is functional and can be activated by androgens, estrogenic, and progestagenic steroids and the nonsteroidal antiandro-

gens hydroxyflutamide and nilutamide (33). In LNCaP cells, AR regulates PSA mRNA and, consequently, PSA protein (34, 35). We therefore incubated LNCaP cells with 50 ng/ml of IL-6 for 24 h and determined PSA mRNA levels afterward by semiquantitative RT-PCR. As shown in Fig. 3, PSA mRNA levels significantly increased after stimulation with IL-6. The mean PSA:β2-microglobulin ratio in IL-6 treated cells was 1.26 ± 0.17 compared with untreated controls that had a mean ratio of 0.67 ± 0.11 (P = 0.012).

Effect of IL-6 on PSA Secretion in LNCaP Cells. After having demonstrated the ability of IL-6 to up-regulate PSA mRNA expression, we measured the production of the androgen-regulated PSA protein in the supernatants of cultured cells after treatment with androgen and/or IL-6. Methyltrienolone provoked a concentration-dependent increase of the secreted PSA protein. Maximal induction was measured with 1 nM R1881. Androgen-independent induction of PSA secretion was observed after treatment with IL-6 (Fig. 4A). Similarly to studies on PSA mRNA, IL-6, at a concentration of 50 ng/ml, caused a ~2-fold increase of PSA protein. This increase in PSA protein secretion was associated with a dose-dependent down-regulation of LNCaP cell proliferation by IL-6. In addition, the R1881-induced proliferative response was diminished in the presence of IL-6, thus confirming previous observations made by Levesque et al. (20). Consistent with our observations in cotransfected DU-145 cells, low doses of androgen (5 and 10 pM) and IL-6 (10 and 25 ng/ml) up-regulated PSA secretion in a synergistic fashion (Fig. 4B). Again, the pure antiandrogen bicalutamide suppressed not only the effects of R1881 on PSA but also those induced by IL-6 alone and in combination with androgen. Experiments in which LNCaP cells were incubated with IL-6 in serum-free medium yielded very similar results (data not shown). These findings confirmed that stimulation of PSA secretion could be attributed to IL-6 itself rather than to low androgen concentrations that might remain in charcoal-stripped FCS.

Effects of PK Inhibitors on PSA Secretion. The ability of inhibitors of PKA and MAPK pathways to down-regulate PSA secretion was tested. PKI and PD 98059 were not toxic for LNCaP cells. Bisindolylmaleimide was excluded from this experiment, because of its toxicity in LNCaP cells. PKI shows only a negligible effect on androgen-induced PSA secretion, and PD 98059 was not effective. In contrast, the PKA inhibitor reduced the effect of IL-6 on PSA secretion by 56%. PD 98059 completely abolished IL-6 stimulation (Fig. 5). These results indicate that PKA and MAPK pathways are involved in the AR-mediated regulation of PSA by IL-6.
DISCUSSION

This study identifies IL-6 as a novel compound that activates the human AR in prostatic carcinoma cells. We also show that this mechanism is not restricted to cells that transiently express the AR (DU-145) but is clearly implicated in the regulation of PSA mRNA and protein expression in a cell line that expresses the endogenous AR (LNCaP). It was demonstrated previously that several polypeptide growth factors, substances that activate PKA, and vitamin D are able to up-regulate AR activity (25–27, 36).

Cross-talk between the IL-6 and AR signaling pathways may be important in carcinoma of the prostate. Abnormally elevated levels of IL-6 in serum were measured in a subset of patients with advanced prostate carcinoma who failed androgen ablation therapy (4). Up-regulation of IL-6 in these patients may be due to the negative regulation of the IL-6 gene by androgenic hormones (37). High levels of IL-6 were found in conditioned media from AR-negative prostate carcinoma cells PC-3, TSU, and DU-145 and primary prostate cancer cultures, whereas contradictory findings as to IL-6 production were reported for LNCaP cells (4, 13, 19). The effects of IL-6 on AR activation were more pronounced in cotransfected DU-145 than in LNCaP cells. This may be explained by higher IL-6 receptor expression in DU-145 cells (13).

The expression of both subunits of the IL-6 receptor gene increases after withdrawal of androgens (38). IL-6 receptor mRNA was detected by Siegmsund et al. (14) in all prostatic carcinomas in their study (14). All these findings emphasize the importance of a cross-talk between IL-6 and androgen signaling pathways in prostatic carcinoma. Expression of AR protein was unequivocally demonstrated in all primary prostate cancers, relapsed tumors, and in their metastases (22–24). Mutant ARs detected in prostate cancer tissues are activated frequently by androgens, other steroids, and even by nonsteroidal AR antagonists (reviewed in Ref. 39). Future studies will probably use some of these mutants to obtain more information on molecular mechanisms responsible for IL-6 activation of the AR. Low concentrations of androgen are present in sera of patients who receive hormone ablation therapy for prostate cancer. Our results show that low doses of androgen and IL-6 activate the AR in a synergistic manner. Consequently, the concentration of androgen that is needed for maximal activation of the AR is reduced by a factor of 100 in the presence of IL-6. Kokontis et al. (40) demonstrated that LNCaP cells gradually adapt to an environment with low androgen supply by increasing transcriptional activity of the AR. The effects of IL-6 on AR activity may therefore be potentiated after long-term androgen ablation.

Ligand-independent activation of the AR, as assessed in reporter gene assays, was reported in this and in three previous studies (25–27). The human AR is similar to the human ER regarding nonsteroidal activation (28, 41). In the case of the AR, there is a series of reports demonstrating androgen-like effects of nonsteroidal substances in a physiological context (42, 43). In human prostate explants, the PKA activator forskolin caused a significant increase in PSA secretion, and its effect was abolished by AR antagonists (44). These results are in line with our findings, and androgen-like effects of IL-6 will probably be further investigated in other in vitro and in vivo models.

The effects of nonsteroidal activators of the AR on prostate cancer cell growth may be either positive or negative. Polypeptide growth factors and substances that stimulate PKA pathway cause stimulation of growth of prostatic epithelial cells (18, 45, 46). In contrast, vitamin D, a potent inhibitor of prostate cancer cell proliferation, similarly to IL-6, increases the secretion of PSA. The effects of vitamin D on PSA were also blocked by bicalutamide (36). In our experiments, a dose-dependent inhibition of proliferation of LNCaP cells by IL-6 was observed. Our results as to the IL-6 effect on LNCaP proliferation are in concordance with those of several other investigators (16, 18, 20). Ritchie et al. (18) demonstrated that IL-6 inhibits the proliferation of DU-145, PC-3, and LNCaP cells. IL-6, which activates the ER, also...

Fig. 4. Regulation of PSA secretion in LNCaP cells by either 1R181 or IL-6 (A) and by R181 and IL-6 (B) in the absence (B) or presence of bicalutamide (B). The cells were incubated in the presence of substances indicated for 72 h. Basal PSA protein levels (B) were set as 100%. Four independent experiments were performed; bars, SE; *, P < 0.05, IL-6 treatment versus untreated control, Mann-Whitney U test.

Fig. 5. Inhibition of IL-6-induced PSA secretion by PK inhibitors. LNCaP cells were pretreated with one of the inhibitors indicated for 30 min and incubated with IL-6 for 72 h. The results are mean values of four experiments; bars, SE.
inhibits the proliferation of ER-positive breast cancer cells (47). However, autocrine and paracrine growth-promoting effects of IL-6 on the three prostate cancer cell lines were reported recently (17, 21). The effect of IL-6 on tyrosine phosphorylation of the growth factor receptor ErbB2 and MAPK activation was demonstrated to be associated with growth stimulation of LNCaP cells (21). One of the reasons for these divergent results may be differences in the levels of expression of intracellular kinases in LNCaP sublines used in various laboratories. In our study, however, IL-6 stimulated MAPK pathways and caused growth inhibition in LNCaP cells. It is recognized that MAPK activation may be associated with both growth stimulation and inhibition, depending on cell type, duration of treatment, and availability of downstream enzyme targets. It was reported that IL-6 decreases proliferation and activates MAPK pathways in MG-63 human osteoblastic cells (48).

IL-6 effects on AR activity in both DU-145 and LNCaP cell lines were blocked by the nonsteroidal antiandrogen bicalutamide. This finding is in agreement with a number of observations that AR antagonists inhibit nonsteroidal activation (25–27, 42–44, 49). In contrast, several PR and glucocorticoid receptor antagonists acquire agonistic properties in the presence of PK activators (50, 51). Thus, the AR is unique among steroid receptors because acquisition of agonistic characteristics due to nonsteroidal activation has never been reported for antiandrogens.

Our experiments in which prostate cancer cells were incubated with IL-6 and PK inhibitors provided some information about the intermedium-signaling events between the IL-6 receptor and the AR. The inhibitors of PKA, PKC, and MAPKs down-regulated reporter gene activity induced by IL-6 in our experiments. These findings provide comprehensive evidence that PKA, PKC, and MAPK pathways are required for AR activation by IL-6. Moreover, a partial inhibition of androgen-induced reporter gene activity was measured with PKI. Our observation as to the ability of PKI to diminish androgen-induced reporter gene activity is consistent with that of Nazareth and Weigel (26). It suggests a regulatory role for PKA in both steroidal and nonsteroidal activation of the AR. In LNCaP cells, which express the endogenous AR, PKI was not effective in inhibition of androgen-induced PSA secretion. The reasons for differences in action of PKI in transfected and nontransfected cells are not clear at present. Inability of PKI to reverse stimulatory effects of androgen on PSA protein secretion in prostate explants was reported by Nakhaia et al. (44).

In summary, the results of our study clearly show that intracellular signaling of IL-6 interacts with the androgen signaling pathway. This communication between signaling pathways results in the up-regulation of AR activity and prostate-specific protein expression. In view of the fact that prostate cancers express IL-6, IL-6 receptors, and the AR, the interactions described in the present study may have clinical significance. Further investigations will probably focus on characterization of this cross-talk in other prostate carcinoma models.

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