Prostate Cancer Cells (LNCaP) Generated after Long-Term Interleukin 6 (IL-6) Treatment Express IL-6 and Acquire an IL-6 Partially Resistant Phenotype

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ABSTRACT

Purpose: The levels of interleukin-6 (IL-6) are frequently elevated in sera from patients with advanced prostate carcinoma. Our main objective was to investigate changes in responsiveness to IL-6 and/or androgen that occur in LNCaP cells after long-term treatment with IL-6. This in vitro model could be of clinical relevance because of its similarity with late-stage prostate carcinoma.

Experimental Design: LNCaP human prostate cancer cells were treated with IL-6 at a concentration of 5 ng/ml. After 20 passages, the new subline LNCaP-IL-6+ has been established. Passages 20–40 are referred to as low passages (LP) and passages 41–73 as high passages (HP). LNCaP cells passaged at the same time in the absence of IL-6 were used as controls (LNCaP-IL-6−). Cells were counted after treatment with either IL-6 or the synthetic androgen methyltrienolone (R1881), and cell cycle analysis was performed. Binding of IL-6 or R1881 was assessed by radioligand binding assays. Reporter gene activity was measured by chloramphenicol acetyltransferase assay. Prostate-specific antigen in LNCaP-IL-6+ supernatants was measured by an enzyme immunoassay. Expression of IL-6 mRNA and protein was assessed by reverse transcription-PCR and ELISA, respectively.

Results: The basal proliferation rate in HP LNCaP-IL-6+ cells was higher than that in LNCaP-IL-6− cells. IL-6 inhibited proliferation of LNCaP-IL-6− cells but not that of either LP or HP of LNCaP-IL-6+ cells. This inability to elicit a growth-inhibitory response was associated with lack of effect on cell cycle distribution in the LNCaP-IL-6+ subline. In parallel, IL-6 binding decreased gradually during long-term IL-6 treatment and, in HP, reached only 33% of the levels measured in controls. Binding of radiolabeled androgen increased 2-fold in HP LNCaP-IL-6+ cells. Reporter gene assays revealed that R1881, at nanomolar concentrations, was a more potent androgen receptor activator in LNCaP-IL-6− than in LNCaP-IL-6− cells. However, androgen- and IL-6-induced prostate-specific antigen secretion decreased in long-term IL-6-treated cells. IL-6 cDNA fragments were detected by reverse transcription-PCR in HP LNCaP-IL-6+ cells but not in controls or LP. IL-6 protein was first detected in passage 36 of LNCaP-IL-6+ cells, and it increased in HP.

Conclusions: Long-term treatment of LNCaP human prostate cancer cells with IL-6 leads to abolishment of inhibitory growth response. In contrast to control cells, the LNCaP-IL-6+ subline expresses IL-6 mRNA and protein.

INTRODUCTION

IL-6, a Mr 21,000–28,000 protein that is produced by a variety of mesenchymal and epithelial cells is a major mediator of inflammation and immunological reactions. Moreover, there is a growing body of evidence demonstrating that IL-6 acts directly on benign and malignant cells. The nature of these responses depends on the cell type and tumor differentiation status. Cell lines derived from early-stage melanoma were growth-inhibited by IL-6, whereas cells representing more advanced stages were resistant to the growth-inhibitory effect (1). In breast cancer, estrogen receptor-positive cells are inhibited by IL-6 in a paracrine manner. In contrast, growth of cells that do not express the estrogen receptor was not affected by IL-6 (2). IL-6 effects in target tissues are determined by the presence of the IL-6R. The IL-6R complex is composed of the ligand-recognizing subunit gp 80 (α-subunit) and the signal-transducing subunit gp 130 (β-subunit), which consists of an extracellular region, a membrane-spanning region, and a cytoplasmic region. After binding of the ligand to the gp 80 subunit, gp 130 subunits homodimerize and activate Janus kinases, which in turn phosphorylate gp 130 (3). After tyrosine phosphorylation of gp 130, STAT family members associate with gp 130 and act as signal transducers and activators of transcription (STAT). The abbreviations used are: IL, interleukin; IL-6R, IL-6 receptor; IL-6, 3 a M r 21,000–28,000 protein that is produced by a variety of mesenchymal and epithelial cells is a major mediator of inflammation and immunological reactions. Moreover, there is a growing body of evidence demonstrating that IL-6 acts directly on benign and malignant cells. The nature of these responses depends on the cell type and tumor differentiation status. 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substrates for Janus kinases. STAT factors translocate to the nucleus as dimers and bind to DNA sequences of promoters of target genes, thus activating their transcription. IL-6 signaling may also involve the Ras protein and activation of the mitogen-activated PK pathway.

For several reasons, IL-6 is considered an important growth-regulatory factor in human prostate cancer. The initial observation that IL-6 serum levels are elevated in a subgroup of patients with metastatic prostate cancer (4) was confirmed by others (5–7). Prostate cancer cell lines express the IL-6R (8) and, for all of them, both positive and negative IL-6 effects on proliferation were reported (9–12). In our laboratory, IL-6 induced growth inhibition of LNCaP cells, which were derived from a lymph node metastasis of a patient with prostate cancer (13, 14). Ligand-independent activation of the AR by IL-6 in that cell line resulted in stimulation of PSA gene expression (13). Mori et al. (10) provided evidence that IL-6 causes a G1 growth arrest in LNCaP cells; down-regulates cdk 2, 4, and 6; and induces expression of the cell cycle inhibitor p27. However, some researchers have reported that IL-6 promotes growth of some prostate cancer cells by means of the SV total RNA isolation system (Promega, Madison, WI). Total RNA (500 ng) was used for the first-strand cDNA synthesis with a commercially available kit (Life Technologies, Inc., Paisley, United Kingdom). cDNA fragments of human IL-6 and $\beta$2-microglobulin (housekeeping many). Chloramphenicol and unlabeled acetyl-CoA were obtained from Sigma Chemical Co. (Deisenhofen, Germany). Human recombinant IL-6 and IL-6 ELISA were purchased from R & D Systems (Minneapolis, MN). The transfection reagent Gene Porter was from Gene Therapy Systems (San Diego, CA).

Proliferation of LNCaP Sublines. LNCaP cell sublines were seeded into 6-well plates at a density of 9 $\times$ 10^4 cells/well in RPMI 1640 supplemented with 10% FCS and antibiotics. After 24-h, the cells were supplemented with medium containing 3% CS FCS in the absence or presence of IL-6 or androgen. After 3 or 6 days of incubation, trypsinized cells were counted with a hemocytometer.

Cell Cycle Analysis. LNCaP cells were grown in 6-well plates in the absence or presence of IL-6 for 72 h. Afterward, they were trypsinized, washed twice with sample buffer (1 g glucose/1 l phosphate-buffered saline), and centrifuged at 400 $\times$ g for 10 min. Cell number was assessed and, after centrifugation and removal of the supernatant, 1 ml cold ethanol was added to the pellet. After 20 h of incubation, cells were mixed using a vortex, centrifuged for 5 min, and the supernatant was discarded. Pellets were dissolved in 1 ml of propidium iodide-staining solution (1 mg/ml propidium iodide and 1000 units RNase A in 10 ml of sample buffer). Cell cycle distribution was analyzed with a flow cytometer (FACS Calibur; Becton Dickinson, San Jose, CA), and data were processed with the Cell Quest software (Becton Dickinson).

IL-6 Binding in LNCaP-IL-6+ Cells. LNCaP-IL-6+ and LNCaP-IL-6− cells were grown in 75 cm^2 flasks, trypsinized, and incubated for 2 h at 4°C with $^{125}$I-IL-6 at concentrations ranging between 0.03 and 0.5 nM in the presence or absence of a 200-fold excess of unlabeled IL-6. Pellets were then recovered by centrifugation (3800 $\times$ g; 3 min) and washed with ice-cold medium. Subsequently, pellets were resuspended in 0.5 ml of 1% SDS/100 mM NaOH. Radioactivity was measured using a gamma counter. Cellular protein was determined according to the method described by Bradford (19), and $B_{max}$ and $K_d$ were calculated by Scatchard analysis.

AR Binding Assay. Measurement of binding of $[^{3}$H]R1881 was performed as described previously (20).

Transfections and Reporter Gene Assays. LNCaP-IL-6+ and LNCaP-IL-6− were transiently transfected by lipofection with the reporter CAT gene, which is driven by a promoter consisting of two androgen responsive elements in front of a TATA box. CAT assay was performed after incubation with androgen and/or IL-6 in cell extracts as described elsewhere (21).

Measurement of PSA in LNCaP Supernatants. LNCaP sublines were grown on 24-well plates in medium supplemented with 3% CS FCS in the absence or presence of androgen or IL-6. After 72 h of incubation, PSA was measured in the supernatants by the microparticle enzyme immunoassay Immx (Abbott Laboratories, Abbott Park, IL). PSA levels were normalized according to a cell number.

IL-6 RT-PCR. Total RNA was isolated from prostate cancer cells by means of the SV Total RNA Isolation System (Promega, Madison, WI). Total RNA (500 ng) was used for the first-strand cDNA synthesis with a commercially available kit (Life Technologies, Inc., Paisley, United Kingdom). cDNA fragments of human IL-6 and $\beta$2-microglobulin (housekeeping...
gene used as an internal control) were amplified using the following primers: IL-6 sense (5'-CTCCAGAACAGATTTTGAG-3'), IL-8 antisense (5'-CCCTAAAGCTTCAGAATG-3'), β2-microglobulin sense (5'-ATGCTGGGCTGTAACATGT-3'), and β2-microglobulin antisense (5'-AGAGCTACCTGGAGCAACCT-3'). cDNA (2 μl) was added to 48 μl of PCR mix containing 10 × buffer [10 mM Tris-HCl (pH 8.8), 1.5 mM MgCl2, 50 mM KCl, 0.1% Triton X-100] for DyNAzyme polymerase (Finnzyme, Vienna, Austria), 200 μM dNTPs, 0.5 μM of the respective oligonucleotide, 4% DMSO (for the housekeeping gene), and 1 unit of DyNAzyme II DNA polymerase. Samples were overlaid with 40 μl of mineral oil. PCR was then performed in a TRIO-Thermoblock (Biometra, Göttingen, Germany) according to the following programs: (a) for the amplification of the IL-6 gene: 2 min at 94°C, 35 cycles of 50 s at 95°C, 1 min at 56°C, 15 s at 73°C, and a final extension of 3 min at 73°C; and (b) for the amplification of the β2-microglobulin gene: 2 min at 94°C, 30 cycles of 25 s at 94°C, 10 s at 96°C, 1 min at 57°C, 90 s at 73°C, and a final extension for 2 min at 73°C. Fragments were visualized after electrophoresis in a 2% agarose gel in TAE buffer [0.04 mM Tris, 0.001 mM EDTA (pH 8.0)] prestained with ethidium bromide.

Measurement of IL-6 in the Supernatants of LNCaP Sublines. IL-6 was determined in the supernatants of LNCaP-IL-6+ and LNCaP-IL-6− cells by ELISA following the instructions of the manufacturer.

RESULTS

Regulation of Proliferative Response in LNCaP-IL-6+ Cells. In an attempt to characterize the phenotype of the newly developed cell line, we have initially investigated the proliferative response to either IL-6 or androgen. After 3 or 6 days of treating LNCaP-IL-6+ cells with 10 or 25 ng/ml of IL-6, they were counted (Fig. 1). IL-6, at these concentrations, affects proliferation of human prostate cancer cells, as shown previously (9–11). The basal proliferation rate did not change in LP (data not shown) but was higher in HP LNCaP-IL-6+ cells than that in LNCaP-IL-6− cells (Fig. 1). Inhibition of proliferation was observed only in controls and was similar to that reported previously for parental LNCaP cells (13). In contrast, growth of LNCaP-IL-6+ cells was not slowed down by IL-6. Inability of IL-6 to cause a negative growth effect on long-term IL-6-treated cells was observed in both LP and HP. The synthetic androgen R1881, administered at concentrations between 0.001 and 10 nm, regulated proliferation of LNCaP-IL-6+ cells and controls in a biphasic manner (Fig. 2).

IL-6 Does Not Cause a G1 Cell Cycle Arrest in Long-Term IL-6-treated Cells. To investigate whether alterations in proliferative response occur in association with changes in cell cycle distribution, flow cytometric DNA analysis was performed. In agreement with a previous publication (10), we have also observed an increase in the percentage of cells in the G1 phase from 76.4 to 88.8% after treatment with IL-6 in LNCaP-IL-6− cells (Table 1) together with a decrease in the percentage of cells in S phase. On the contrary, IL-6 did not affect cell cycle distribution in long-term IL-6-treated cells. Most notably, the percentage of cells in G1 and S phase remained unchanged after IL-6 treatment. These data show that the inability of IL-6 to inhibit LNCaP-IL-6+ cells correlates with its failure to elicit a G1 cell cycle arrest.

Reduction of IL-6 Binding in LNCaP-IL-6+ Cells. To study the mechanisms that might be associated with a lack of IL-6 growth inhibition in the new LNCaP subline, we have measured binding of radiolabeled IL-6 in long-term IL-6-treated cells and controls. Maximal binding and dissociation constant were determined by means of a Scatchard plot. IL-6 binding slightly decreased in LP of LNCaP-IL-6+ cells. Maximal decrease in IL-6 binding was achieved in HP as shown in Fig. 3. It was 33% of that in controls. Maximal decrease in IL-6 binding was achieved in HP as shown in Fig. 3. It was 33% of that in controls. There were no differences in Kd between control and long-term treated cells. Thus, failure of IL-6 to induce a negative growth response in LNCaP-IL-6+ cells was associated with a down-regulation of binding sites for IL-6.
Cross-Talk with the Androgen Signaling Pathway.

We (13) and others (22) have demonstrated that the human AR can be activated by IL-6 and that this activation depends on functionality of multiple signaling pathways. In the present study, we have investigated whether prolonged treatment of LNCaP cells with IL-6 affects cross-talk with the androgen-signaling pathway. First, we measured binding of radiolabeled synthetic androgen R1881 in long-term IL-6-treated cells. Scatchard analyses of binding assay results revealed that AR protein expression increased gradually beginning with LP, and a maximal 2-fold increase was observed in HP cells (Fig. 4), whereas $K_d$ remained constant.

LNCaP cells were then transfected with a synthetic androgenic reporter gene, and CAT activity was measured in extracts from cells treated with androgen and/or IL-6. As demonstrated in Fig. 5, there was no difference in AR activity between both cell lines after stimulation with R1881 at concentrations of 10 and 100 pm or IL-6. However, treatment of LNCaP-IL-6+ cells with nanomolar concentrations of methyltrienolone yielded higher CAT activities than those measured in LNCaP-IL-6− cells. IL-6 enhanced AR activity induced by picomolar concentrations of R1881. These results show that even after prolonged IL-6 treatment, which alters the expression of the IL-6R and AR, the cross-talk with the androgen-signaling pathway is preserved.

**Table 1** Cell cycle distribution in IL-6-resistant cells

<table>
<thead>
<tr>
<th>Treatment</th>
<th>LNCP-IL-6−</th>
<th>LNCP-IL-6+ HP</th>
</tr>
</thead>
<tbody>
<tr>
<td>No treatment</td>
<td>G 1</td>
<td>76.4 ± 0.8</td>
</tr>
<tr>
<td></td>
<td>S</td>
<td>8.9 ± 0.5</td>
</tr>
<tr>
<td></td>
<td>G 2</td>
<td>15.2 ± 0.3</td>
</tr>
<tr>
<td>IL-6 (10 ng/ml)</td>
<td>G 1</td>
<td>88 ± 1.8</td>
</tr>
<tr>
<td></td>
<td>S</td>
<td>3.9 ± 1.1</td>
</tr>
<tr>
<td></td>
<td>G 2</td>
<td>8.1 ± 0.7</td>
</tr>
<tr>
<td>IL-6 (25 ng/ml)</td>
<td>G 1</td>
<td>88.8 ± 1.9</td>
</tr>
<tr>
<td></td>
<td>S</td>
<td>3.7 ± 0.8</td>
</tr>
<tr>
<td></td>
<td>G 2</td>
<td>7.6 ± 1.6</td>
</tr>
</tbody>
</table>

* Percentages of cells in each cell cycle phase are given.

Fig. 2 Androgen-induced proliferative response in long-term IL-6-treated (passages 71–72) and control cells. The sublines were grown and counted as described in the legend for Fig. 1a in the absence or presence of the synthetic androgen R1881.

Fig. 3 Specific binding of radiolabeled IL-6 in LNCaP-IL-6+ cells. Long-term IL-6-treated cells and controls were cultured in medium containing CS FCS for 48 h. The cells were scraped off and incubated with increasing concentrations of $^{125}$I-IL-6 for 2 h at 4°C. Nonspecific binding was measured in the presence of a 200-fold molar excess of unlabeled IL-6. After washing, radioactivity was measured in a gamma counter. The results obtained with the passage 70 of LNCaP-IL-6+ cells are shown. $B_{max}$ and $K_d$ were calculated by Scatchard analysis.

Fig. 4 R1881 binding in LNCaP-IL-6+ cells (passage 69). LNCaP-IL-6+ cells and controls were incubated with increasing concentrations of $[^{3}H]$R1881 for 90 min at room temperature. After incubation, the cells were washed twice with ice-cold medium and specific binding was determined.
Regulation of PSA Protein Secretion in LNCaP-IL-6+ Cells. PSA levels in the supernatants of LNCaP-IL-6+ cells were measured after stimulation with either R1881 or IL-6 (Fig. 6). Interestingly, androgen- and IL-6-induced PSA expression decreased 2–3-fold in the LNCaP-IL-6/H11001 subline.

Expression of IL-6 in IL-6 Partially Resistant Cell Lines. RNA was isolated from parental LNCaP cells, LNCaP-IL-6-, and passages 20 (LP) and 73 (HP) of LNCaP-IL-6/H11001 cells and transcribed into cDNA. Whereas β2-microglobulin fragments were amplified from all of the samples, IL-6 could only be amplified from HP LNCaP-IL-6/H11001 (Fig. 7). IL-6 secretion was measured in the supernatants of these cell lines by ELISA. Consistent with previous reports (11, 23), no IL-6 could be detected in the supernatants of parental LNCaP cells. IL-6 secretion was not detectable in the supernatants of LNCaP-IL-6- cells either. IL-6 could be first measured in passage 36 of long-term-treated cells, and higher levels were detected in passage 63 (Table 2).

DISCUSSION

After the initial observation that IL-6 serum level elevation occurs in patients with metastatic prostate cancer (4), several researchers focused on the role of this cytokine in prostate tumors. The present study was designed on the basis of the assumption that continuous stimulation of the IL-6R in patients with metastatic prostate cancer affects tumor cell proliferation, communication with the AR, and expression of endogenous IL-6. Because a new in vitro model was established, it has allowed us to monitor these changes. Newly generated cells were characterized in terms of proliferative response to IL-6 and androgen, cell cycle distribution, expression of IL-6 and the IL-6R, and expression and activation of the AR. In our laboratory (13) and according to some other reports (11, 12, 24, 25), IL-6 inhibited growth of parental LNCaP cells. In contrast, some studies showed a stimulation of growth of LNCaP cells by IL-6 through phosphorylation of the growth factor receptor ErbB2 (9, 16, 26). The reasons for these discrepancies are not clear yet.
and it is hypothesized that differences in cell culture conditions have an impact on the outcome of these experimental studies. In a previous study (10), it was shown that IL-6-induced G1 growth arrest is associated with accumulation of the cell cycle inhibitor p27; down-regulation of cdk 2, 4, and 6; and inhibition of cdk-associated kinase activities. We are currently investigating which of these regulatory mechanisms is altered in the LNCaP-IL-6+ subline. There was no evidence that IL-6 induces apoptosis in the parental cell line. Several phenotypical changes that were notable in HP also appeared even in LP of LNCaP-IL-6+ cells; however, the most remarkable difference between LP and HP cells was that the latter cells secreted considerable amounts of IL-6 into the supernatants. However, these IL-6 levels are lower than those measured previously in the supernatants of androgen-sensitive DU-145 and PC-3 cells (489 and 2202 pg/ml/10^6 cells/72 h, respectively; Ref. 4). Thus, at least in LNCaP cells, progression toward IL-6 resistance might include three steps. Initially, cells are growth-inhibited by IL-6, such as parental LNCaP or LNCaP-IL-6− in our study, and express neither IL-6 mRNA nor protein. Secondly, during prolonged IL-6 treatment, a negative regulation of growth is abolished, but IL-6 expression and secretion are low. In the third step, IL-6-resistant cells that secrete high amounts of IL-6 are generated. The answer to the function of IL-6 in HP LNCaP-IL-6+ cannot be given at present, but several possibilities could be discussed: (a) IL-6 produced by epithelial cells might affect surrounding stromal cells in a paracrine manner; these stromal cells may, in turn, be stimulated toward the production of growth factors; (b) IL-6 effect might be indirect because of modulation of immune response or angiogenesis (27); and (c) the hypothesis that IL-6 in these cells provides an advantage for establishment of an autocrine loop is unlikely because of down-regulation of surface IL-6R, as it is evidenced in the present study. To clarify this issue, additional studies investigating the role of IL-6 in prostate cancer growth in vivo are needed.

At present, it could not be explained why a cell line selected in the presence of exogenous IL-6 acquires the ability to produce that cytokine contrary to normal feedback mechanisms. At least three mechanisms responsible for up-regulation of IL-6 in HP LNCaP-IL-6+ cells should be discussed. Repression of the IL-6 gene expression by androgens and estrogens was reported for bone cell cultures and LNCaP cells (28–30). However, induction of IL-6 mRNA and protein expression in HP LNCaP-IL-6+ cannot be attributed to any change in steroid concentration in the serum in which the LNCaP-IL-6+ cell line was maintained. It was recently shown that IL-6 gene repression is associated with its hypermethylation in breast cancer cells (31). It is also possible that such a mechanism plays a role in parental LNCaP cells. Finally, changes in expression of nuclear factor κB, which is the exclusive transcription factor for induction of the IL-6 gene, might occur in our model system.

Beside prostate cancer, IL-6 acts as a bifunctional cytokine in melanoma. Cell lines derived from early melanoma stages are growth-arrested by IL-6. In contrast, cells obtained from advanced stage melanoma are not susceptible to IL-6 inhibition (1). Interestingly, analysis of binding of [125]IL-6 to the gp 80 subunit did not show any difference between IL-6-sensitive and -nonsensitive cell lines in one study (32), whereas Silvani et al. (33) observed a loss of cytokine binding to the cell surface in IL-6-resistant cells. Mechanisms that lead to reduced binding of IL-6 may include down-regulation of IL-6R mRNA, as evidenced in human monocytes (34), or receptor trapping within intracellular components. Regulation of the IL-6R in LP and HP LNCaP-IL-6+ is currently being investigated in our laboratory. Another similarity between HP LNCaP-IL-6+ and advanced stage melanoma cells is the fact that they both express and secrete IL-6.

Although IL-6 binding in LNCaP-IL-6+ cells is diminished, IL-6 was equally efficient in this cell line in activation of the AR, as in LNCaP-IL-6− cells. Therefore, we do not consider the newly generated subline completely nonresponsive to IL-6. One possible explanation is the fact that the AR is expressed at a higher level in cells generated after long-term IL-6 treatment. Alternatively, activity of intracellular PK systems might not be strictly dependent on expression levels of the IL-6R. We have demonstrated that signaling pathways of PKA, mitogen-activated PK, and PKC are required for functional activation of the AR by IL-6 in prostate cancer cells (13). The AR regulates genes responsible for regulation of proliferation, such as CDK (35) and cell cycle inhibitors (36). PSA is up-regulated by IL-6 in LNCaP cells because of activation of the AR (13, 22). On the basis of measurement of AR activity in LNCaP-IL-6+ cells, one could expect similar PSA values compared with those from LNCaP-IL-6− cells. However, it was demonstrated in two independent studies that AR up-regulation does not parallel with enhanced expression of PSA in the course of long-term androgen ablation in LNCaP cells (37, 38). The PSA gene promoter might be hypermethylated during either prolonged androgen deprivation or IL-6 treatment.

We showed previously that IL-6 and the IL-6R could be detected in nearly all of the tumor tissues from prostate cancer patients (23). Therefore, we believe that the changes in their expression and function described in the present study may have an impact on the clinical situation. Similarly to IL-6, TGF-β is a bifunctional regulator of prostate cancer cells (reviewed in Ref. 39). Prostate cancer cell lines derived from metastases show loss of growth inhibition by TGF-β in contrast to those derived from primary tumors. They secrete great quantities of TGF-β into the supernatants (40). Receptors for TGF-β are often not detectable in advanced prostate cancer (41). The TGF-β system is, in this respect, different from the IL-6 signaling pathway. The IL-6R is obviously present in specimens from prostate cancer patients (23).

We point to IL-6 as a good candidate target for therapy in prostate cancer because of its association with morbidity (4), significance as a prognostic factor (42), antiapoptotic effects in some prostate cancer cells (18), protection of cell death induced by chemotherapeutic agents (43), and lack of inhibition of tumor cell growth as reported in this study. Therefore, it should be tested whether interference with IL-6 signaling provides some therapeutic benefits in prostate cancer.

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REFERENCES


Partial Resistant Phenotype Interleukin 6 (IL-6) Treatment Express IL-6 and Acquire an IL-6 Partially Resistant Phenotype

Alfred Hobisch, Reinhold Ramoner, Dietmar Fuchs, et al.