

Interleukin-6 stimulation of growth of prostate cancer *in vitro* and *in vivo* through activation of the androgen receptor

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Abstract

It is hypothesized that ligand-independent activation of the androgen receptor is one of the mechanisms implicated in tumour progression. However, supportive evidence is limited to the effect of HER-2/neu that stimulates prostate cancer progression through activation of the androgen receptor. In the present study, we have asked whether the proinflammatory cytokine interleukin-6 (IL-6), which is known to stimulate androgen receptor activity and expression of its downstream target genes, may also induce growth of androgen-sensitive cells. We have found that IL-6 differentially regulates proliferation of LAPC-4 and MDA PCa 2b cells. In MDA PCa 2b cells, growth stimulation by IL-6 was reversed by administration of either the non-steroidal anti-androgen bicalutamide or the inhibitor of the mitogen-activated protein kinase pathway PD98059. Neither cell line was found to express endogenous IL-6. Interestingly, the treatment of those prostate cancer cells did not increase phosphorylation of STAT3. The effect of IL-6 on stimulation of androgen receptor activity in MDA PCa 2b cells was lower than that of androgen, comparable with findings reported by other researchers. However, growth of MDA PCa 2b xenografts in castrated animals treated with IL-6 was similar to that in non-castrated animals. In addition, bicalutamide showed an inhibitory effect on IL-6-regulated growth *in vivo*. Taken together, data in the present study demonstrate that IL-6 may cause growth of androgen receptor-positive tumours *in vitro* and *in vivo* through activation of the androgen receptor.

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Introduction

Prostate cancer whose growth is regulated largely through activation of the androgen receptor (AR) is the most common malignancy in the western world in men. Most small tumours detected by prostate-specific antigen (PSA) screening are nowadays cured by radical prostatectomy and radiation therapy. By contrast, patients with non-organ-confined tumours receive palliative treatment with androgen ablation or blockade of the AR (Huggins *et al.* 1941). This therapy is effective for an average of 3–5 years before tumours regrow and metastasize to bone and visceral organs.

The AR is a ligand-mediated transcription factor that belongs to the superfamily of steroid receptors. They

have similar structures containing the N-terminal domain (NTD) that harbours activation function-1 (AF-1), the DNA-binding domain, hinge region and the ligand-binding domain (LBD) in which AF-2 is located. After ligand binding, the complex between a ligand and receptor is formed. Following translocation to the nucleus, the ligand-AR complex binds to specific androgen response elements, interacts with coactivators and modulates the expression of androgen-regulated genes.

Several mechanisms implicated in prostate cancer progression are AR-related. They involve AR gene amplification and overexpression, mutations or ligand-independent activation. A number of mutations in AR

gene are located in the LBD (Marcelli *et al.* 2000, Buchanan *et al.* 2001) and are responsible for broadened ligand specificity. For example, mutated AR Met⁷¹⁵ and Ala⁸⁷⁷ are increasingly activated by adrenal androgens, androgen metabolites, oestrogenic steroids, progesterone and flutamide (Culig *et al.* 1993, Suzuki *et al.* 1993).

It is established that, either in the absence of ligands or in the presence of low androgenic concentrations, AR can be stimulated by growth factors, proinflammatory cytokines (Culig *et al.* 1994, Hobisch *et al.* 1998, Chen *et al.* 2000), forskolin (Nazareth & Weigel 1996, Wang *et al.* 2006), bombesin (Lee *et al.* 2001) or HER-2/neu (Craft *et al.* 1999). One of the cytokines whose serum levels are elevated in patients with refractory prostate cancer is interleukin-6 (IL-6). It is a 28 kDa protein that belongs to the IL-6 type cytokines family. It has an effect on cell proliferation, apoptosis and angiogenesis through activation of Janus kinase (Jak)-signal transducer and activators of transcription (STAT) factor 3 (Heinrich *et al.* 2003), mitogen-activated protein (MAP) kinase (Ernst *et al.* 1996) and phosphoinositol 3-kinase (PI3-K)-Akt (Chen *et al.* 1999a,b) signalling pathways. IL-6 can affect the growth of prostate cancer cells in a paracrine and autocrine manner. Previous experimental work investigating the ability of IL-6 to induce AR transactivation revealed enhanced expression of an androgen-sensitive reporter gene caused by IL-6 that was inhibited by the non-steroidal anti-androgen bicalutamide (Hobisch *et al.* 1998). Although, many studies have been performed to analyze the mechanisms of AR ligand-independent activation, its functional relevance for tumour growth is still under investigation. It was demonstrated by Craft *et al.* (1999) that cross-talk between HER-2/neu, a member of the epidermal growth factor (EGF) receptor tyrosine kinase family, and the AR may be a determinant of tumour progression. Experiments with non-castrated and castrated mice inoculated with LNCaP cells over-expressing HER-2/neu showed that this oncogene can substitute for androgen and cause tumour growth *in vivo*.

In this study, we sought to clarify the relevance of AR activation by IL-6 for tumour growth *in vitro* and *in vivo*. The androgen-sensitive MDA PCa 2b prostate cancer cell line expresses mutated (L701H and T877A) AR and is derived from a single bone metastasis from a patient with a relapsed tumour (Navone *et al.* 1997). MDA PCa 2b cells were applied in our studies and stimulatory effect of IL-6 on their proliferation is shown for the first time. To better characterize the responsiveness to IL-6, the androgen-dependent

LAPC-4 prostate cancer cell line derived from a lymph node metastasis that expresses wild-type AR was examined as well (Klein *et al.* 1997).

Materials and methods

Reagents

The synthetic androgen methyltrienolone (R1881) was purchased from R&D Systems (Minneapolis, MN, USA) and dissolved in absolute ethanol. Human recombinant IL-6 was provided by R&D Systems and the working solution was prepared in PBS. Bicalutamide was a kind gift from Astra Zeneca (Macclesfield, UK). PD98059 was purchased from VWR (Vienna, Austria). Stock solutions of bicalutamide and PD98059 were prepared in dimethyl sulfoxide (DMSO). 5-azacytidine was obtained from Sigma and dissolved in acetic acid:water (1:1 v/v).

Cell culture

The LAPC-4 prostate cancer cell line was a kind gift from Prof. Charles Sawyers (formerly at University of California, Los Angeles, CA, USA). The cells were cultured in Iscove's modified Dulbecco's (IMDM) medium (PAA Laboratories, Pasching, Austria) containing 15% FCS (Szabo Scandic, Vienna, Austria), 100 U/ml penicillin, 100 U/ml streptomycin solution and 10 nM R1881.

The MDA PCa 2b prostate cancer cell line was obtained from Szabo Scandic. The cells were grown in F12 medium (Sigma-Aldrich) supplemented with 20% FCS, 100 U/ml penicillin, 100 U/ml streptomycin solution, 25 ng/ml cholera toxin (Sigma-Aldrich), 10 ng/ml EGF (Strathmann Biotech, Hannover, Germany), 5 μ M phosphoethanolamine, 100 pg/ml hydrocortisol and insulin/transferin/selenid mix (Invitrogen).

To investigate the responsiveness of prostate cancer cells to IL-6 after prolonged exposure to the cytokine, LAPC-4 and MDA PCa 2b were maintained under regular culture conditions in the presence of 5 ng/ml IL-6 during a period of 3 weeks. After this period, new sublines named LAPC-4-IL-6 and MDA PCa 2b-IL-6 were generated and used in proliferation assays.

The AR negative CV-1 cell line derived from monkey kidney was obtained from ATCC (Rockville, MD, USA). CV-1 cells were cultured in DMEM (PAA Laboratories) supplemented with 10% FCS, 100 U/ml penicillin and 100 U/ml streptomycin. All cell lines were maintained at 37 °C in a humidified 5% CO₂ atmosphere.

³H-Thymidine uptake assay

LAPC-4, LAPC-4-IL-6, MDA PCa 2b and MDA PCa 2b-IL-6 cells were seeded at a density of 10^4 cells/well onto 96-well plates in regular culture media. Serum requirements for optimal growth were determined for each cell line and subline. The next day, media were changed and the cells cultured either without (MDA PCa 2b IL-6) or with 5% steroid-free FCS (LAPC-4, LAPC-4-IL-6, MDA PCa 2b), 100 U/ml penicillin and 100 U/ml streptomycin. Cells were incubated with increasing doses of IL-6 (1–50 ng/ml). After incubation, the mix of $1 \mu\text{l}$ ³H-thymidine (1 μCi /well) and 49 μl serum-free medium/well was added. Cells were then harvested after 24 h and DNA was fixed on filter plates (UniFilter, Perkin Elmer, Boston, MA, USA). Chameleon 5025 liquid scintillation counter (HVD Life Sciences, Vienna, Austria) was used to read the filter plates.

Cell counting

MDA PCa 2b cells were seeded at a density of 5×10^5 cells/well onto six-well plates in regular culture medium. They were incubated with IL-6 (25 ng/ml), bicalutamide (5 μM) or PD98059 (25 μM) for 72 h. The cells were trypsinized, centrifuged and cell pellet was suspended in 1 ml PBS. 100 μl cell suspension in PBS was added to 10 ml Casy Ton buffer (Innovatis, Reutlingen, Germany). The cell number based on the cell size was counted by the instrument (Schaerfe System, Reutlingen, Germany).

Reverse transcription PCR

LAPC-4 and MDA PCa 2b cells were incubated for 48 h in the absence or presence of the demethylating agent 5-azacytidine (1–5 μM). RNA was isolated according to the manufacturer's instructions (Qiagen). The procedure of cDNA reverse transcription with Super Script III RT (Invitrogen) was based on the manufacturer's protocol. The IL-6 gene fragment was amplified by PCR with the primers: sense, 5'-CCT CCAGAACAGATTTGAGA-3'; antisense, 5'-CCT TAAAGCTGCGCAGAATG-3' under the following conditions: 2 min at 94 °C, 30 cycles of 50 s at 95 °C, 1 min at 56 °C, 15 s at 73 °C, with the final extension time of 3 min at 73 °C. The gp80 gene fragment was amplified with the primers: sense, 5'-TCGCCAGTA GTGTCGGGAGCA-3'; antisense, 5'-GCCTTCCTT CAGAGCCCCGAG-3' and the gp130 gene fragment was amplified with the primers: sense, 5'-CCACAT ACGAAGACAGACCA-3'; antisense, 5'-GCGTTC TCTGACAACACACA-3' under the following

conditions: 15 min at 95 °C, 35 cycles of 50 s at 95 °C, 1 min at 60 °C, 40 s at 73 °C, with the final extension time of 3 min at 73 °C. The fragments were visualized using 2% agarose gel electrophoresis.

IL-6 ELISA

MDA PCa 2b and LAPC-4 cells were seeded onto six-well plates at a density of 2.5×10^5 cells/well in regular culture media and let to attach and reach the subconfluence. The cells were treated with TNF α (1–5 ng/ml) or IL-1 β (0.1–0.5 ng/ml) for 72 h. After the incubation period, the supernatant from the cells was collected and used for further investigations. Cells were harvested by trypsinization and counted by Casy cell counter. Human IL-6 ELISA (Bender System, Vienna, Austria) assay was performed according to the manufacturer's instructions. The results of the assay were normalized according to the cell number.

Western blot analysis

LAPC-4 and MDA PCa 2b cells were seeded in culture media and let to grow to subconfluence. After 24 h incubation, the cells were maintained in fresh serum-free medium. Cells were incubated with increasing concentrations of IL-6 between 5 and 45 min and then harvested and lysed with European Organization for Research and Treatment of Cancer (EORTC) lysis buffer by incubation on ice for 20 min. The lysed cells were centrifuged 13 000 r.p.m. for 10 min and the supernatant (cytosolic fraction) was collected. The amount of protein was measured by Bradford method (Bradford 1978) and 20–50 μg protein were used for SDS-PAGE analysis. Polyacrylamide 4–12% gradient Bis-Tris gel (Invitrogen) was run in $1 \times$ MOPS (Invitrogen) buffer at 150 V. Transfer of the proteins to PVDF membrane (Invitrogen) was done in $1 \times$ transfer buffer (Invitrogen) containing 10% methanol at 30 V for 1 h. Membrane was blocked in Starting Block buffer (Pierce Biotechnology, Rockford, IL, USA) for 1 h at room temperature and then incubated with either phospho-specific p44/p42 MAP kinase (Thr 202/Tyr 204) E10 mouse monoclonal antibody (New England Biolabs, Beverly, MA, USA), p44/p42 rabbit polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA), phospho-specific STAT3 (Y 705, clone 9E12) mouse monoclonal antibody (Cell Signalling, Danvers, MA, USA), STAT3 (C-20) rabbit polyclonal antibody (Szabo Scandic), Jak1 (413104) rat monoclonal antibody (R&D Systems), Jak2 (691R5) mouse monoclonal antibody (Abcam, Cambridge, UK), phospho-specific Akt (Ser 473) rabbit polyclonal antibody (Cell Signalling) or Akt

(C-20) goat polyclonal antibody (Santa Cruz) overnight. The fluorescent signals were read using the Odyssey infrared imaging system (LiCor Biosciences, Lincoln, NE, USA).

Transfections

CV-1 cells were seeded onto 24-well plates at a density of 10^5 cells/well in regular culture medium. After 24 h the medium was changed and transfection with Fugene 6 (Roche) was performed in serum-free conditions according to the manufacturer's instructions. Cells were transiently transfected with the reporter construct ARE₂-TATA-luc (pGL3-E) and either wild-type (pSG5-AR) or mutated (L701H and T877A) AR expression vector (pSG5-AR_{L701H,T877A} was a kind gift from Prof. David Feldman, Stanford University School of Medicine, Stanford, CA, USA). We used 400 ng DNA per well with the ratio between reporter gene and AR of 20:1. The plasmid pGL4.73 encoding renilla gene (30 ng) was used as a transfection efficiency control.

In vivo experiments

Three to four weeks old male nude mice (*nu/nu* BALB/c) were purchased from Charles River Laboratories (Sulzfeld, Germany) and allowed to acclimate for 1 week. All procedures involving mice were approved by Austrian Federal Ministry of Science and Research (GZ BMWF-66.011/0076-C/GT/2007). MDA PCa 2b cells suspended in 50 μ l PBS and mixed with the same volume of Matrigel (BD Biosciences, Two Oak Park, Bedford, MA, USA) were injected subcutaneously into the right flank of all mice ($n=10$; 6×10^6 cells per mouse; Fig. 1). Tumour size was measured using a calliper and tumour volume was calculated according to the formula: length \times width² of a tumour area/2. The tumours were harvested after they reached the mean volume of 1200 mm³ and passed from mouse to mouse. A new group of mice ($n=22$) was inoculated with tumour tissues (300 mm³ per mouse). After tumours reached the mean volume of 400 mm³, mice were grouped as follows: 1) non-castrated ($n=8$), 2) castrated and treated with IL-6 ($n=8$), 3) castrated and treated with IL-6 and bicalutamide ($n=6$). Orchiectomy was performed under anaesthesia using ketamine-HCl and xylazine-HCl (100 mg/kg mouse i.p. and 10 mg/kg mouse i.p. respectively). To inhibit an inflammatory response, 4 mg/kg mouse of ketoprofen s.c. during the first day after castration was used. Treatment with 200 ng IL-6 i.p. twice per day and 50 mg/kg mouse of bicalutamide i.p. twice per week was initiated immediately (Culig et al. 1999,

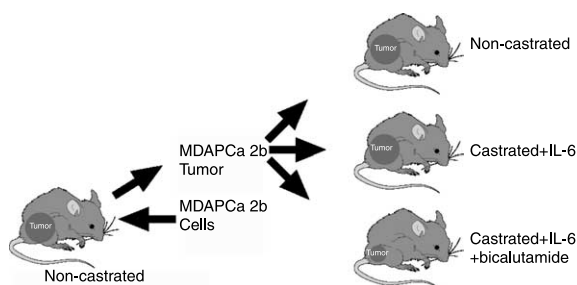


Figure 1 Schematic overview of *in vivo* experiments.

Wang et al. 2004). General condition of animals allowed performance of experiments until day 38. The mice were killed by cervical dislocation.

Statistical analysis

The SPSS 12.0 program was used for statistic evaluation and the Mann–Whitney *U*-test was used for the assessment of statistical significance.

ANOVA test was applied to evaluate statistical significance of the tumours' volume between non-castrated versus IL-6-treated mice and IL-6-treated mice versus IL-6 and bicalutamide-treated mice.

Results

Proliferative response of LAPC-4 and MDA PCa 2b cells to IL-6 treatment

In order to characterize the interactions between IL-6 and the AR, we initially studied the growth response of two AR-positive cell lines to IL-6 treatment. We observed a dose-dependent significant growth inhibition of LAPC-4 cells after 48 h (Fig. 2A), an effect similar to that previously reported in parental LNCaP cells (Degeorges et al. 1996, Mori et al. 1999). In MDA PCa 2b cells, increasing doses of IL-6 enhanced proliferation (Figs 2B and 3). We have further examined whether the inhibition of AR activity by the anti-androgen bicalutamide has an effect on IL-6-induced proliferation of MDA PCa 2b cells. The proliferation of MDA PCa 2b cells was inhibited in the presence of IL-6 and bicalutamide compared with IL-6 treatment alone. PD98059, an inhibitor of the MAP kinase pathway, abolished the effect of IL-6 on growth stimulation (Fig. 3).

To verify whether long term treatment with IL-6 has opposite effects on cell proliferation to transient treatment, we established new sublines of LAPC-4 and MDA PCa 2b cells, named LAPC-4-IL-6 and MDA PCa 2b-IL-6. Continuous exposure of LAPC-4 cells to IL-6 did not alter the pattern of the response to

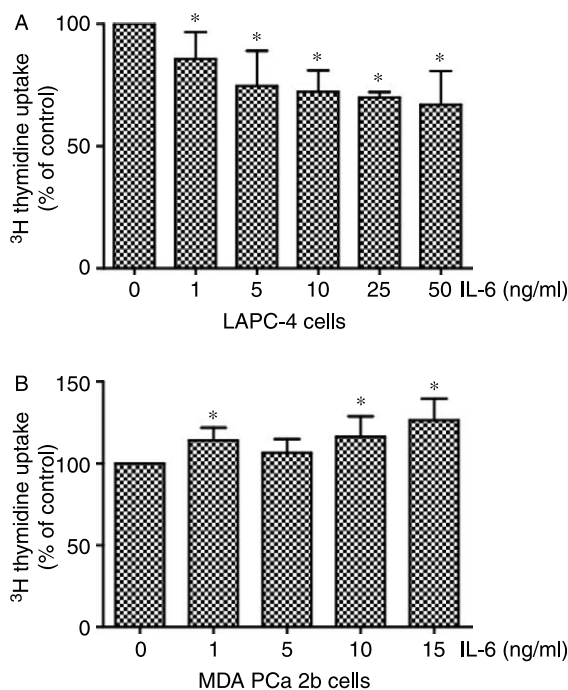


Figure 2 ³H-thymidine incorporation assay after treatment with increasing doses of IL-6 for 48 h in (A) LAPC-4 and (B) MDA PCa 2b cells. Data represent the mean \pm s.d. $n=3$. * $P<0.05$ versus control, Mann–Whitney U -test.

increasing doses of IL-6 and inhibition of proliferation was still observed (Fig. 4A). Basal proliferation rate of the LAPC-4-IL-6 subline was similar to that of the parental LAPC-4. Interestingly, in the MDA PCa 2b-IL-6 subline a significant increase in proliferation was achieved with higher doses of IL-6 (25 and 40 ng/ml) than in parental MDA PCa 2b cells (Figs 2B and 4B). Long term treatment of MDA PCa 2b cells with IL-6 markedly enhanced basal proliferation in comparison to the parental cell line. Additionally, we did not

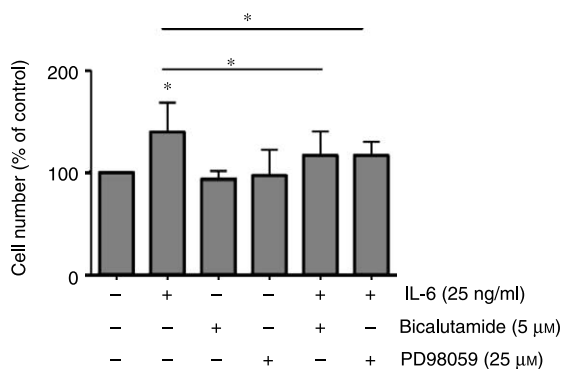


Figure 3 Effects of bicalutamide or PD98059 in the presence of IL-6 in MDA PCa 2b cells. Data represent the mean \pm s.d. $n=3$. * $P<0.05$ versus control, Mann–Whitney U -test.

observe apparent changes in morphology and phenotype of LAPC-4-IL-6 and MDA PCa 2b-IL-6 sublines by phase contrast microscopy (data not shown).

Expression of IL-6 and IL-6R in LAPC-4 and MDA PCa 2b cells

In this set of experiments, we examined if IL-6 affects LAPC-4 and MDA PCa 2b cells proliferation in a paracrine or autocrine fashion. To investigate this issue, RT-PCR and ELISA for IL-6 were performed. In contrast to IL-6-positive PC-3 cells, IL-6 mRNA was not detected in either LAPC-4 or MDA PCa 2b cell line growing under basal conditions (Fig. 5). Promoter hypermethylation was considered as a possible mechanism involved in IL-6 gene suppression. However, a 48 h treatment of cells with the demethylating agent 5-azacytidine did not induce the expression of the cytokine (Fig. 5). To confirm the absence of IL-6 secretion, conditioned media from LAPC-4 and MDA PCa 2b cells were subjected to ELISA. IL-6 was not secreted by either untreated cells or those treated with TNF. α or IL-1 β , known to act as inducers of expression of the IL-6 gene (data not shown; Kohase *et al.* 1986, Kitamura *et al.* 1998).

On target cells, IL-6 acts via a receptor complex composed of a ligand-binding 80 kDa glycoprotein (gp80, IL-6R) and a 130 kDa signal-transducing receptor subunit (gp130). The investigated cell lines expressed both subunits of the receptor complex as detected by RT-PCR (Fig. 6). Taken together with the results of proliferation experiments, these data imply that IL-6 regulates growth of both cell lines in a paracrine manner.

Lack of STAT3 phosphorylation in the LAPC-4 and MDA PCa 2b cell lines after IL-6 treatment

The Jak-STAT pathway is one of the classical signalling pathways activated by IL-6 that may be implicated in carcinogenesis in different ways. It has been shown that IL-6 induces transcriptional activity of STAT3 in parental LNCaP cells (Spiotto & Chung 2000). To understand the role of STAT3 in IL-6-regulated growth of LAPC-4 and MDA PCa 2b cells, western blot analyses of phosphorylated and total STAT3 were performed. STAT3 protein was detected in both cell lines. We measured the levels of STAT3 phosphorylation at tyrosine residue 705 in cells treated with IL-6 at different time points after 24 h of serum starvation. As shown in Fig. 7, we did not observe a constitutive or IL-6-induced STAT3 phosphorylation in either cell line. In parental LNCaP cells that were used as a control, the addition of 10 ng/ml IL-6 entailed

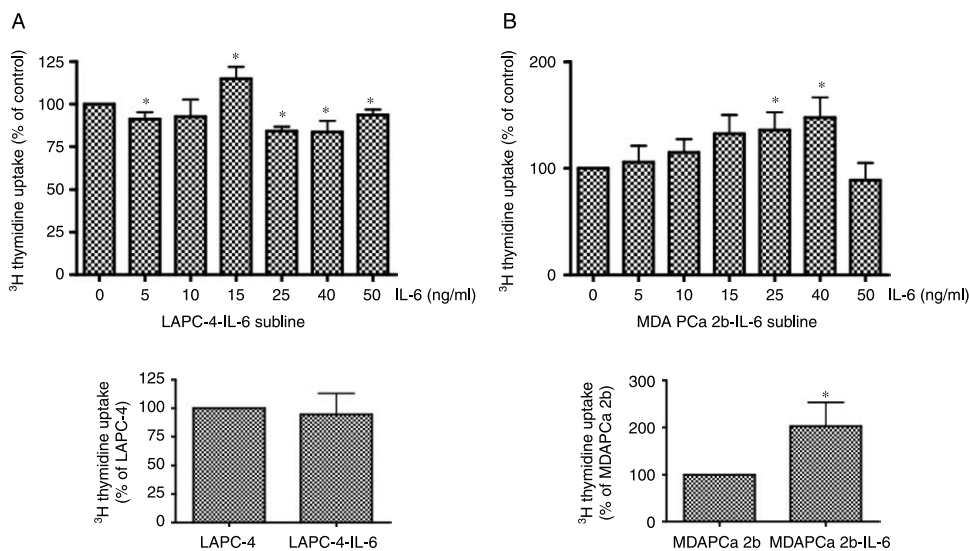


Figure 4 Regulation of growth of prostate cancer cell sublines established after long term treatment with IL-6. ³H-thymidine incorporation assay after treatment with increasing doses of IL-6 in (A) LAPC-4-IL-6 and (B) MDA PCa 2b-IL-6 sublines. Basal proliferation of (A, lower panel) LAPC-4-IL-6 and LAPC-4 set as 100% and (B, lower panel) MDA PCa 2b-IL-6 and MDA PCa 2b set as 100% is shown. Data represent the mean \pm s.d. $n=3$. * $P<0.05$ versus control, Mann-Whitney U -test.

an increase in the phosphorylated form of STAT3 (Godoy-Tundidor *et al.* 2005). The lack of STAT3 phosphorylation raised a question about the expression of signalling intermediates such as Jak1 and Jak2. In both studied cell lines, constitutive expression of Jak1 was observed (Fig. 8A). Interestingly, Jak2 was detected in MDA PCa 2b but not in LAPC-4 cells (Fig. 8B).

Phosphorylation of MAP kinases in LAPC-4 and MDA PCa 2b cells following IL-6 treatment

Activation of p44/p42 MAP kinase after treatment with IL-6 is mostly associated with increased proliferation (Nakafuku *et al.* 1992, Daeipour *et al.* 1993). To further analyze IL-6 signal transduction pathways, we examined the effect of IL-6 on p44/p42 phosphorylation. After 15 min of incubation, IL-6 (10 ng/ml) significantly elevated phosphorylation of p42 in MDA PCa 2b cells (Fig. 9A). IL-6, at concentrations of 10 or 25 ng/ml, increased the amount of the phosphorylated form of p44 (Fig. 9A). However, it should be mentioned that the basal levels of phosphorylated p44/p42 in the MDA PCa 2b cell line were elevated, comparable with those previously observed in androgen-insensitive cell lines (Putz *et al.* 1999). By contrast, we did not observe any phosphorylation of p44/p42 in LAPC-4 cells in the absence or presence of IL-6 (Fig. 9B). The results correlated with those from proliferation assays, suggesting a role of the MAP kinase pathway in the growth stimulation of MDA PCa 2b cells.

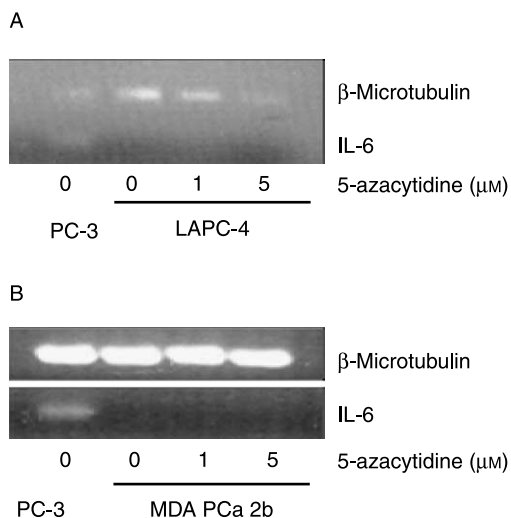


Figure 5 Analysis of expression of IL-6 in (A) LAPC-4 and (B) MDA PCa 2b cells. IL-6 mRNA was not detected by RT-PCR in cells growing under basal conditions or after treatment with the demethylating agent 5-azacytidine. PC-3 cells were used as a positive control.

Phosphorylation of Akt in the LAPC-4 and MDA PCa 2b cell lines after IL-6 treatment

As established before, the PI3-K-Akt pathway may be activated after IL-6 treatment (Chen *et al.* 1999a). To test phosphoregulation of Akt by IL-6, LAPC-4 and MDA PCa 2b cells were subjected to IL-6 stimulation

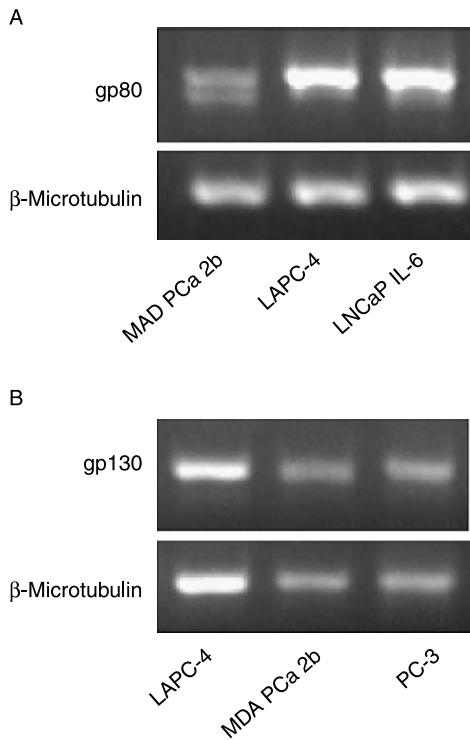


Figure 6 Analysis of expression of gp80 and gp130 in prostate cancer cells by RT-PCR. LNCaP-IL-6- and PC-3 cells were used as positive controls respectively.

with doses up to 25 ng/ml at several time points. Interestingly, 25 ng/ml IL-6 induced a significant upregulation of phosphorylated Akt in LAPC-4 cells after 15 min (Fig. 10A). Western blot analysis revealed the lack of the activated form of Akt in MDA PCa 2b cells under basal culture conditions and after treatment with IL-6 (Fig. 10B).

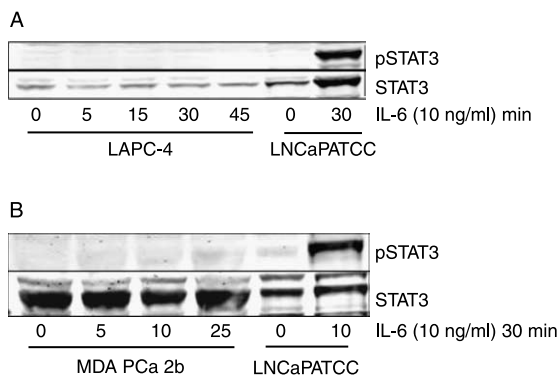


Figure 7 Lack of STAT3 phosphorylation in prostate cancer cells LAPC-4 and MDA PCa 2b. After treatment of (A) LAPC-4 and (B) MDA PCa 2b cells with IL-6, we did not observe phosphorylation of STAT3 in contrast to LNCaP ATCC cells in which IL-6 caused an increase in phosphorylated STAT3.

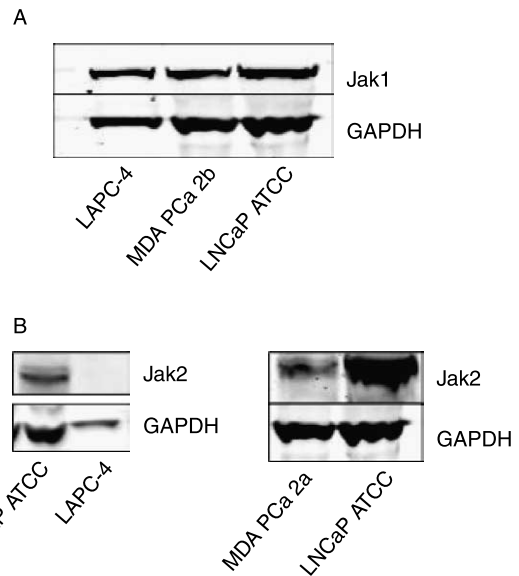


Figure 8 Expression of Jak1 and Jak2 in prostate cancer cells. (A) Jak1 expression was detected in both cell lines. (B) The lack of Jak2 was observed in LAPC-4 in contrast to MDA PCa 2b cells. LNCaP ATCC cells were used as positive controls.

Activation of wild-type and mutated AR

Since the MDA PCa 2b cell line is in our experimental conditions, the only AR-positive cell line that responds to IL-6 treatment by growth stimulation, we have selected those cells for the *in vivo* experiment. Prior to initiation of the *in vivo* study, we verified whether IL-6 activates the mutated AR. To compare activation potency between the wild-type AR and the AR carrying the double mutations (L701H and T877A), we used CV-1 cells in which either receptor and androgen-responsive reporter were transiently expressed. Cells cotransfected with the wild-type AR showed a high induction of reporter gene expression by androgen (13.9-fold; Fig. 11A). In comparison with the wild-type AR, a reduced effect on expression of luciferase gene was noted for the mutated form of AR (Fig. 11B). These findings are in accordance with the report of Zhao *et al.* (1999). The action of androgen was diminished by bicalutamide in cells with either wild-type (Fig. 11A) or mutated AR (Fig. 11B). Bicalutamide itself exerted a partial stimulatory effect on AR activity, consistent with results reported by others (Hara *et al.* 2003). In our experimental system, IL-6 was able to slightly increase the expression of reporter gene in the presence of mutated AR (Fig. 12).

The role of IL-6 in tumour progression *in vivo*

On the basis of our proliferation assays with MDA PCa 2b cells, we hypothesized that IL-6 is able to induce

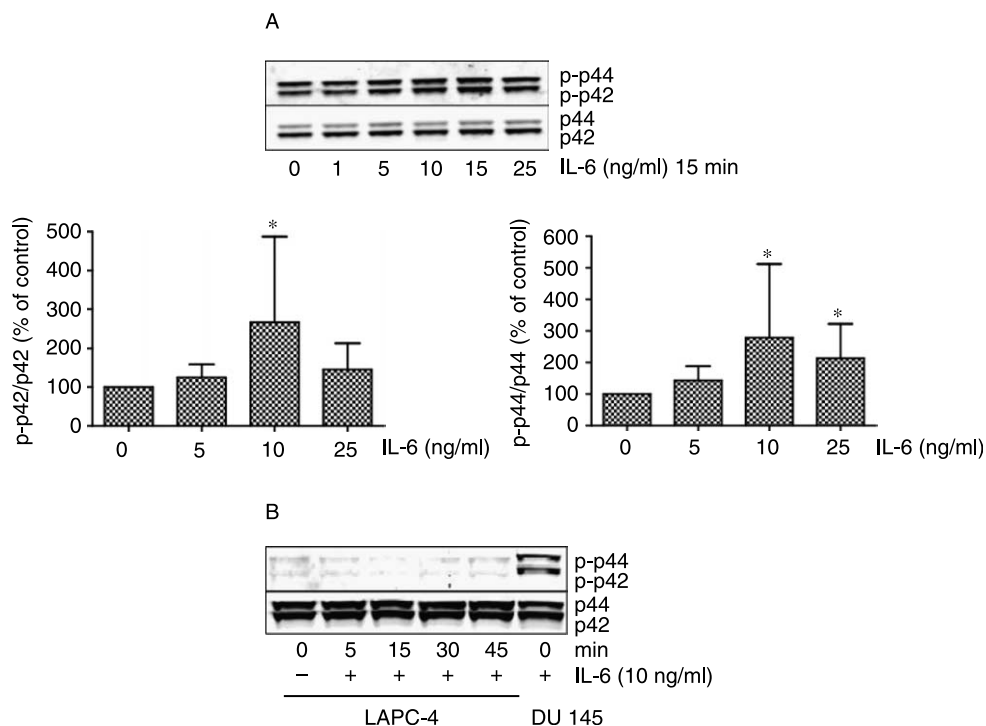


Figure 9 Effect of IL-6 on activation of the MAP kinase pathway. IL-6 significantly increased MAP kinase phosphorylation in (A) MDA PCa 2b cells in which the basal levels of phosphorylated p44/p42 are elevated. Data represent the mean \pm s.d. $n=3$. * $P<0.05$ versus control, Mann-Whitney U -test. (B) IL-6 does not affect p44/p42 phosphorylation in LAPC-4 cells. DU-145 cells were used as a positive control.

tumour growth *in vivo*. To address the main question of the present study, i.e. whether IL-6 promotes prostate tumour growth through AR activation, MDA PCa 2b cells were xenografted into nude mice. To obtain the tumorigenic MDA PCa 2b cells, we passed them on non-castrated *nu/nu* immune-deficient mice. After 10 weeks, we observed tumour growth and used those tumour tissues for inoculation in another group of *nu/nu* immune-deficient mice. In the non-castrated animals, the tumour volume reached 1119 mm³ after 5.5 weeks (Fig. 13), whereas in castrated animals that received daily IL-6 treatment, the mean tumour volume was found to be 990 mm³ at the end of the experiment (Fig. 13). By contrast, tumours did not significantly grow in castrated IL-6 and bicalutamide-treated mice (Fig. 13). These results showed that the action of IL-6 is antagonized by bicalutamide *in vivo* thus providing new insights about the significance of interactions between IL-6 and the AR in regulation of prostate cancer growth (Figs 1 and 13).

Discussion

It has been hypothesized that ligand-independent activation of the AR is one of the mechanisms

implicated in prostate tumour progression. So far most of the studies have reported the results on AR regulation by a number of non-steroidal compounds. In those studies, transactivation assays in heterologous cell lines were performed. However, little is known about specific cellular events that are regulated by interactions between most activators of the AR and the receptor itself. IL-6 is a multifunctional cytokine that enhances AR activity in the absence of ligand and is increasingly expressed in prostate cancer tissue in early stages. IL-6 levels higher than 7 pg/ml correlate with bad prognosis (Nakashima *et al.* 2000). Prostatic tumours typically metastasize to bone, lymph nodes and liver, organs in which IL-6 is present. For these reasons, investigations on regulation of growth of androgen-dependent xenografts by IL-6 may be of high clinical relevance.

The key finding of our study is that, IL-6 similarly to HER-2/neu can substitute for androgen in stimulation of AR-positive tumour growth *in vivo*. It is, however, not clear to which extent the implications of our results could be generalized. We selected MDA PCa 2b cells for the *in vivo* study since two other AR-positive cell lines, LNCaP or LAPC-4 is growth-inhibited *in vitro*, as evidenced in the present and previous publications

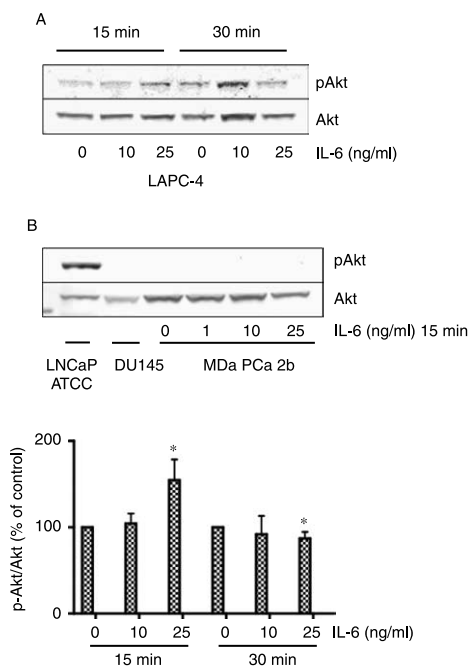


Figure 10 (A) Treatment of LAPC-4 cells with 25 ng/ml IL-6 caused an increase of the phosphorylated form of Akt. (B) By contrast, no phosphorylation of Akt in MDA PCa 2b cells was observed. Data represent the mean \pm s.d. $n=3$. * $P<0.05$ versus control, Mann–Whitney U -test. LNCaP ATCC and DU-145 cells were used as positive or negative controls, respectively.

(Hobisch *et al.* 2001). Moreover, *in vivo* growth of LNCaP cells was suppressed by IL-6 as published by Wang *et al.* (2004). It should be, however, mentioned that contrasting effects of IL-6 in LNCaP cells *in vitro* were reported. These variances may be explained by recent findings according to which phosphorylation of STAT3 is dependent on cell density in melanoma (Kreis *et al.* 2007). If this phenomenon is also relevant to prostate cancer models, one could better understand why divergent results on growth regulation and STAT3 phosphorylation were obtained with LNCaP cells. The role of STAT3 in development and progression of several malignancies has been well-documented (Horiguchi *et al.* 2002). Although, there are many examples demonstrating that STAT3 exerts oncogenic features, its action as an inhibitor of tumour growth has also been reported (Moran *et al.* 2008). One emerging concept supported by our own data is that the anti- or proapoptotic role of STAT3 is determined by the presence of suppressor of cytokine signalling-3 (SOCS-3). SOCS-3 expression was not induced by IL-6 in cells in which phosphorylation of STAT3 is associated with growth retardation and differentiation (Bellezza *et al.* 2006). Interestingly, we reported the

lack of pSTAT3 in LAPC-4 or MDA PCa 2b cell lines under basal conditions and after IL-6 incubation. Signalling of IL-6 involves intermediate molecules like Jak1 or Jak2. Both LAPC-4 and MDA PCa 2b cells express Jak1, whereas Jak2 is present only in MDA PCa 2b cells. On the basis of studies performed in other cell lines, diverse mechanisms could be considered for explanation of lack of phosphorylation of STAT3. In a number of plasma cell lines, STAT3 phosphorylation in response to IL-6 was detected although Jak1 protein is not expressed (Kopantzev *et al.* 2002). Those authors also demonstrated that IL-6 may fail to induce phosphorylation of Jak2 in plasma cells. Finally, STAT3 phosphorylation might be influenced by the MAP kinase pathway in different ways. Studies performed with the herbal remedy magnolol in endothelial cells support the view that Jak-independent mechanisms also regulate STAT3 phosphorylation (Chen *et al.* 2006). Magnolol suppressed IL-6 effect on STAT3 phosphorylation without causing an inhibition of Jak activation.

Diverse growth factors, cytokines and protooncogene products activate the small G protein Ras and subsequently the MAP kinase network (Papatsoris *et al.* 2007). MAP kinase is also a classical pathway for IL-6 and its activation is frequently associated with either reduced apoptosis or, more commonly, enhanced proliferation (Culig *et al.* 2005a). Ras and Raf mutations are not frequent in prostate cancer (Dong 2006) and it could be hypothesized that wild-type Ras and/or Raf are activated by growth factors in an autocrine and paracrine manner during prostate carcinogenesis. It has been suggested that activation of Ras through the MAP kinase pathways modulates AR-dependent gene expression (Bakin *et al.* 2003). Previous studies revealed that AR function is influenced by Ras/MAP kinase interactions with AR-associated proteins (Culig *et al.* 2005b). Elevation of PSA levels is also a p44/p42-sensitive phenomenon in androgen-independent conditions (Franco *et al.* 2003). MDA PCa 2b cells after incubation with IL-6 showed an increase in phosphorylation of p44/p42 kinases. Steiner *et al.* (2003) reported that the functional MAP kinase pathway is at least partly responsible for enhanced growth of cells generated after long term IL-6 treatment. MDA PCa 2b is another example of a cell line in which IL-6-induced MAP kinase activity is associated with growth stimulation. In parental LNCaP cells whose growth is inhibited by IL-6, there is no constitutive MAP kinase activity (Chen *et al.* 1999b, Putz *et al.* 1999). We did not observe phosphorylation of p44/p42 in LAPC-4 cells that respond to IL-6 exposure by inhibition of

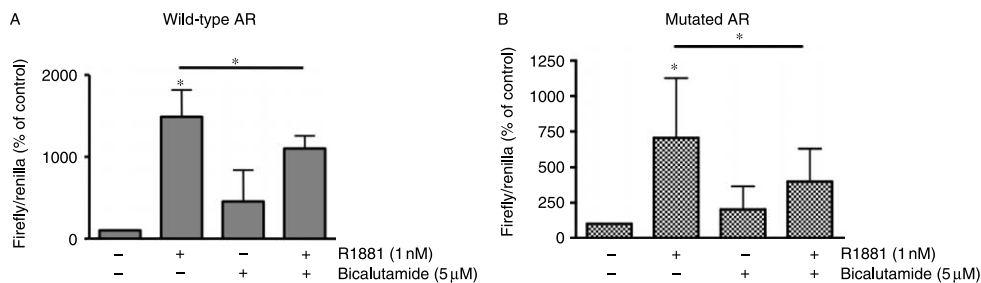


Figure 11 Transcriptional activity of either (A) wild-type or (B) MDA PCa 2b mutated AR after treatment with the synthetic androgen methyltrienolone (R1881). The effects of androgen were blocked by the anti-androgen bicalutamide. Data represent the mean \pm s.d. $n=4$ (wild-type AR), $n=5$ (mutated AR). * $P<0.05$ versus control, Mann-Whitney U -test.

proliferation. All these observations implicate that there is an activation of the MAP kinase pathway in cell lines whose proliferation is increased by IL-6. A similar effect on MAP kinase phosphorylation was found with IL-8, a cytokine that also causes AR activation. Its modest effect on proliferation of LNCaP cells was demonstrated by Seaton *et al.* (2008).

IL-6 treatment can cause activation of the PI3-K-Akt pathway in prostate cancer cells. Yang *et al.* (2003) demonstrated suppression of AR activity by IL-6 via the PI3-K-Akt. Functional significance of IL-6-induced Akt phosphorylation in LAPC-4 cells could not be explained in the present study. One could expect that activation of that pathway will decrease apoptosis. In this particular case, a possibility that pAkt action on downstream genes is antagonized by endogenous Akt inhibitors may be considered.

Following chronic treatment with IL-6, prostate cancer cells can alter the responsiveness to the cytokine. LNCaP cells acquired the ability to proliferate at a higher rate and become more tumorigenic (Hobisch *et al.* 2001, Steiner *et al.* 2003). Our novel sublines were generated after the time period of 3 weeks, similarly to the approach by Sortino *et al.* (2000) who exposed prostate cancer cells to the nerve

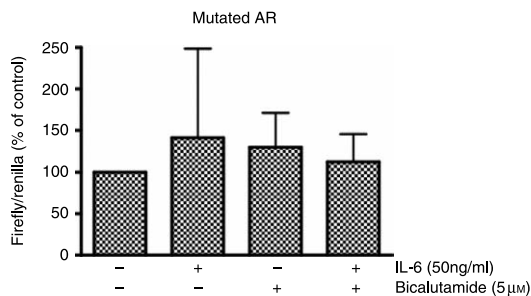


Figure 12 IL-6 effect on activation of the mutated AR. Luciferase assays were performed in CV-1 cells cotransfected with reporter gene and mutated AR expression vector after treatment with IL-6.

growth factor treatment for 2 weeks. The new subline MDA PCa 2b-IL-6 acquired a growth advantage after IL-6 exposure in contrast to LAPC-4-IL-6 cells, thus suggesting that the regulation previously observed in a LNCaP subline may be of general relevance (Hobisch *et al.* 2001).

IL-6 significantly induced the growth of MDA PCa 2b xenografts in nude mice. The stimulation of tumour formation by IL-6 appears to be a result of AR activation by IL-6 signalling because the non-steroidal anti-androgen bicalutamide antagonizes this effect. It should be kept in mind that there is a clear ligand-independent pro-differentiation effect of IL-6 on expression of the AR-target gene PSA in LNCaP cells (Ueda *et al.* 2002a). Two recent publications have also addressed the issue of biological significance of non-steroidal AR activation in prostate disease. Importantly, Wang *et al.* (2006) demonstrated that expression of only a limited number of genes is targeted by cross-talk between androgen and the protein kinase A (PKA) pathway. On the basis of reporter gene studies, one could expect a higher

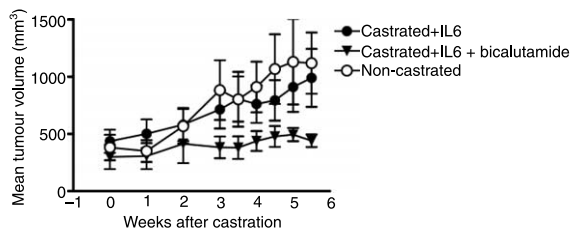


Figure 13 Growth of MDA PCa 2b tumours in male nude *nu/nu* immune-deficient mice. MDA PCa 2b cells were injected into non-castrated or castrated male nude mice. Castrated animals were treated with IL-6 or IL-6 and bicalutamide. Tumour size was measured once weekly. Data represent the mean \pm s.d. $n=8$ (non-castrated), $n=8$ (castrated + IL-6-treated), $n=6$ (castrated + IL-6 + bicalutamide-treated). Significance between the animals' groups was calculated with ANOVA ($P<0.001$, IL-6 treatment versus IL-6 and bicalutamide treatment; $P<0.01$, control versus IL-6 and bicalutamide treatment).

Table 1 Responsiveness of androgen receptor (AR)-positive prostate cancer cells to interleukin-6 (IL-6) and activation of signalling pathways

	LAPC-4	LNCaP	MDA PCa 2b
IL-6 effect on proliferation	Inhibition	Inhibition	Stimulation
Androgen dependency	Dependent	Independent	Independent
Androgen sensitivity	Sensitive	Sensitive	Sensitive
Activation of signalling pathways after IL-6 exposure			
MAP kinase	No phosphorylation	Basic phosphorylation	Increased phosphorylation
STAT3	No phosphorylation	Increased phosphorylation	No phosphorylation
Akt	Increased phosphorylation	Increased phosphorylation Wegiel <i>et al.</i> (2008)	No phosphorylation
SOCS-3	Present	Absent	?
Ligand-independent AR activation and tumour formation	ErbB2 IL-6 not known	IL-6 inhibits	IL-6 stimulates
AR	Wild-type	T877A	L701H and T877A

number of genes to be similarly regulated by androgen and a compound that activates the PKA pathway. In case of AR activation by the neuropeptide bombesin, it was shown that the addition of bicalutamide to cells stimulated by bombesin causes a negative growth effect (Desai *et al.* 2006). In addition, 72 genes, most of which are transcription factors and signal transduction proteins, were found to be similarly regulated by androgen and bombesin.

AR activation by IL-6 was first described by Hobisch *et al.* (1998) and similar results were confirmed by other investigators (Debes *et al.* 2002). Previous experiments were carried out either in cells transfected with an AR expression vector or in LNCaP cells that transiently expressed various reporter genes. Variances in levels of reporter gene expression between reports could be a consequence of utilization of different systems to detect AR activity. The levels of reporter gene expression greatly varied, however, the role of the NTD and the MAP kinase pathway in AR activation by IL-6 seems to be established. Since there is no mutation in the NTD of the MDA PCa 2b AR, it is also not surprising that the effects of IL-6 were detected. We hypothesize that the opposite proliferative responses of AR-positive prostate cancer cells to IL-6 treatment are due to differences in expression of AR coregulators rather than the presence of receptor mutation(s). It has been shown that SRC-1 and p300 cofactors are required for AR activation by IL-6 (Debes *et al.* 2002, Ueda *et al.* 2002b). In the presence of a p300 mutant that lacks histone acetylase activity, the effect of IL-6 was greatly diminished (Debes *et al.* 2002). Under experimental conditions reported by Ueda *et al.* (2002a), the levels of AR activity in LNCaP induced by IL-6 were much lower than those stimulated by androgen. Nevertheless, the same

authors also showed that IL-6 could induce expression of an AR-regulated gene. Those previous results are similar to our data obtained with the MDA PCa 2b AR. Various AR mutations were found in human prostate cancer. It was of our interest to elucidate distinct activation potency of the wild-type AR and the AR with double mutation (L701H and T877A) expressed in LAPC-4 and MDA PCa 2b cells respectively. We confirmed previous findings showing that the wild-type AR was stimulated by androgen to higher levels than the mutated one (Zhao *et al.* 1999).

We demonstrated that neither MDA PCa 2b nor LAPC-4 cells express endogenous IL-6. The lack of IL-6 gene expression was also reported in LNCaP cells, whereas AR-negative PC-3 and DU-145 cells secrete high amounts of the cytokine into the supernatants (Chung *et al.* 1999). This inverse correlation could be explained by the presence of the AR, since it was shown in bone cell cultures that steroids inhibit expression of IL-6 (Bellido *et al.* 1995). With aging, serum androgen levels decline while serum IL-6 levels increase (Li *et al.* 1993). A body of evidence showed that IL-6 gene can be induced by nuclear factor kappa B (NFκB). NFκB regulates expression of genes involved in cell growth and survival. It has been reported that androgen has an inhibitory effect on NFκB (Keller *et al.* 1996) and levels of NFκB are increased in androgen-independent xenografts in comparison to androgen-dependent ones (Chen & Sawyers 2002). Further studies may be performed in order to investigate whether NFκB is inactive in MDA PCa 2b and/or LAPC-4 cells.

Because of its pleiotropic role and increased expression, IL-6 is considered a potential target for therapy in a subgroup of prostate cancer patients. Most studies were carried out with the chimeric monoclonal

anti-IL-6 antibody CNTO 328. Steiner *et al.* (2006) showed a partial reduction in tumour growth in nude mice inoculated with LNCaP-IL-6+ cells after CNTO 328 treatment. This effect may be at least in part a consequence of inhibition of expression of the anti-apoptotic Mcl-1 gene (Cavarretta *et al.* 2007). Similar data were obtained when PC-3 and LuCaP 35 xenografts were treated with CNTO 328 (Smith & Keller 2001, Wallner *et al.* 2006). Future therapy approaches using the anti-IL-6 antibody may be developed with MDA PCa 2b xenografts grown in the presence of IL-6.

In summary, the major finding of this paper is that IL-6 is able to maintain tumour growth through activation of the AR. Our results suggest that stimulation of *in vivo* growth by IL-6 may be relevant in a subgroup of prostate cancer patients. Complex regulation of cellular events by IL-6 in AR-positive cell lines is summarized in Table 1. The findings of the present study may therefore improve understanding how IL-6 affects cellular events, such as tumour cell growth *in vitro* and *in vivo* through its interaction with the AR pathway and allow development of a more effective experimental therapy based on inhibition of IL-6 action in prostate cancer.

Declaration of interest

The authors declare no conflict of interest.

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Author contribution statement

Kamilla Malinowska – performed *in vitro* studies including proliferation, signal transduction experiments, PCR, reporter gene assays, analyzed data, wrote the first version of the manuscript and corrected it on the basis of discussions with other group members.

Hannes Neuwirt – assisted in proliferation studies, prepared a legal request for animal experiments, designed and performed animal experiments, analyzed data, commented on all versions of the manuscript.

Iliaria T Cavarretta – provided advice for *in vitro* experiments, analyzed data, commented on several versions of the manuscript.

Jasmin Bektic – performed animal experiments and commented on the first version of the manuscript.

Hannes Steiner – performed animal experiments and commented on the first version of the manuscript.

Hermann Dietrich – designed animal experiments, analyzed data and commented on several versions of the manuscript.

Patrizia L Moser – collected material from animal experiments, analyzed data and commented on the first version of the manuscript.

Dietmar Fuchs – performed IL-6 measurements, analyzed data and commented on the first version of the manuscript.

Alfred Hobisch – participated in conception of the study, data analysis and commented on the first version of the manuscript.

Zoran Culig – planned the study, led group meetings in which data were discussed, supervised experimental work, corrected drafts of the paper and prepared it for submission.

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