Acquisition of Agonistic Properties of Nonsteroidal Antiandrogens after Treatment with Oncostatin M in Prostate Cancer Cells[1]

Sonia Godoy-Tundidor, Alfred Hobisch, Karina Pfeil, Georg Bartsch, and Zoran Culig[2]

Department of Urology, University of Innsbruck, A-6020 Innsbruck, Austria (S. G-T., A. H., K. P., G. B., Z. C.), and Department of Urology, General Hospital Feldkirch, A-6800 Feldkirch, Austria (A. H.)

ABSTRACT

Purpose: **Interleukin-6 (IL-6), a proinflammatory cytokine the serum and tissue levels of which are elevated in prostate cancer patients, activates the androgen receptor (AR) in a ligand-independent and synergistic manner. Oncostatin M (OSM) is an IL-6 type cytokine that regulates the growth of prostate cancer cells in a paracrine fashion. The present study was designed to investigate the regulation of AR expression and function by OSM, as well as the efficacy of the nonsteroidal antiandrogens hydroxyflutamide and bicalutamide in the inhibition of AR-mediated signal transduction.**

Experimental Design: **Expression of the OSM receptor-β in the prostate cancer cell lines LNCaP, PC-3, and DU-145 was investigated by reverse transcription-PCR. DU-145 and PC-3 cells were cotransfected with an androgen-responsive gene and AR cDNA. Reporter gene activity was measured after treatment with androgen and/or OSM in the absence or presence of antiandrogens or protein kinase inhibitors. AR expression after OSM treatment was assessed by Western blot.**

Results: **OSM receptor-β expression was higher in DU-145 and PC-3 than in LNCaP cells. OSM caused ligand-independent activation of the AR in DU-145 cells, and the maximal activation was 62% of that induced by the synthetic androgen methyltrienolone. In the presence of OSM, hydroxyflutamide behaved as an AR agonist. Bicalutamide down-regulated AR activation caused by OSM only at a concentration of 1 μM. The inhibitor of the protein kinase A signaling pathway PKI and dn signal transducers and activators of transcription (STAT) 3 showed no effect on AR activation by OSM. The inhibitor of the MAPK pathway, PD 98059, caused only a minor down-regulation of OSM-induced reporter gene activity. OSM did not change AR expression in DU-145 cells transfected with AR cDNA.**

Conclusions: **OSM is a member of the IL-6 family of cytokines, which causes ligand-independent activation of the AR without altering receptor expression. In contrast to AR activation by IL-6, nonsteroidal AR antagonists act as agonists in the presence of OSM. This may be attributable to recruitment of different intermediary signal transduction proteins by OSM and IL-6, respectively. The acquisition of agonistic properties of AR blockers in the presence of OSM might compromise use of these drugs in prostate cancer treatment.**

INTRODUCTION

Prostate cancer is the most commonly diagnosed neoplasm in men in industrialized countries. Despite the earlier diagnosis, prostate tumor incidence is increasing. Prostate cancer growth in early and intermediate stages depends on androgenic stimulation and endocrine therapy is, therefore, aimed at the inhibition of androgen signaling. Unfortunately, tumor progression, which could be detected by increased levels of serum prostate-specific antigen, occurs in most patients. The AR[3] plays a central role in the regulation of proliferation, differentiation, and apoptosis of prostate cancer cells. The AR is up-regulated in therapy-resistant prostate cancer, as demonstrated in immunohistochemical studies and real-time PCR experiments (1, 2). The AR can be activated, even in the absence of androgens, by substances that increase intracellular cAMP levels or by growth factor receptor-mediated events (3, 4). AR transactivation potential may be increased through the activation of MAPK, as evidenced in cases of receptor stimulation by HER-2/neu (5).

There is increasing evidence for the existence of a cross-talk between the signaling pathways of IL-6 and the AR in prostate cancer cells (6–9). For several reasons, interaction between the two signaling pathways is clinically significant: (a) serum IL-6 levels are elevated in patients suffering from metastatic carcinoma of the prostate (10, 11); (b) IL-6 and its receptor were detected in prostate cancer cells on immunohistochemistry (12); (c) IL-6 concentration is increased even in tissues from patients with clinically localized prostate cancer (13); and (d) IL-6 exhibits different effects on prostate cancer cell lines, varying from the induction of G_1 growth arrest to the inhibition of apoptosis (14, 15). There is limited information available on biological effects of other IL-6-type cytokines,

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[2] To whom requests for reprints should be addressed, at Department of Urology, University of Innsbruck, Anichstrasse 35, A-6020 Innsbruck, Austria. Phone: 43-512-504-4818; Fax: 43-512-504-4817 or 43-512-504-8365; E-mail: Zoran.Culig@uibk.ac.at.

[3] The abbreviations used are: AR, androgen receptor; MAPK, mitogen-activated protein kinase; IL-6, interleukin-6; OSM, oncostatin M; LIF, leukemia inhibitory factor; OSMR, OSM receptor; dn, dominant-negative; STAT, signal transducers and activators of transcription; db cAMP, dibutyryl cAMP; PK, protein kinase; EGF, epidermal growth factor; RT-PCR, reverse transcription PCR; CAT, chloramphenicol acetyltransferase.

namely OSM, LIF, ciliary neurotrophic factor, neurotrophin-1, and cardiotrophin-1, in prostate cancer cells. Signaling of all these cytokines is mediated through the shared signal transducing subunit gp130, which explains a certain redundancy on the actions exerted by the members of this family. The M_r 28,000 glycoprotein OSM is the only one that can interact with two different kinds of receptor complexes, namely the gp130/ OSMRβ and gp130/ LIF receptorβ. After receptor activation and oligomerization, the Jak/STAT and the MAPK pathways become activated. Subsequently, STAT factors translocate to the nucleus and initiate transcription of acute-phase response genes. Mori *et al.* (16) demonstrated that OSM acts as a paracrine growth factor for the hormone-refractory prostate cancer cell line DU-145. DU-145 cells express the specific gp130/ OSMRβ but do not secrete detectable amounts of OSM, the major source of which are activated monocytes and T-lymphocytes. We asked whether paracrine stimulation of prostate epithelium by OSM, which occurs in prostate cancer, affects AR activity. The present study was, therefore, designed to investigate the regulation of AR expression and function by OSM, as well as the efficacy of clinically used nonsteroidal AR antagonists in the inhibition of AR-mediated signal transduction. The alterations of antagonist/agonist balance of AR blockers are clinically important because of their relationship with prostate cancer progression.

MATERIALS AND METHODS

Cell Culture. LNCaP, PC-3, and DU-145 cells were grown in RPMI 1640 (Hy-Clone, Logan, UT) supplemented with 10% FCS and penicillin/streptomycin (PAA Laboratories, Linz, Austria) and incubated at 37°C in a 5% CO_2 atmosphere.

Chemicals. Human recombinant OSM, the cAMP analogue db cAMP, and the PKA inhibitor PKI were purchased from Sigma (Deisenhofen, Germany). The synthetic androgen methyltrienolone (R1881) was from New England Nuclear (Dreieichenhain, Germany). The nonsteroidal antiandrogen hydroxyflutamide was from Essex Pharma (Munich, Germany), and bicalutamide was synthesized at Schering AG (Berlin, Germany). The MAPK pathway inhibitor PD 98059 was provided by Merck Eurolab (Darmstadt, Germany), and human recombinant EGF was from Haidu MediTec (Vienna, Austria).

RT-PCR. Total RNA from hormone-refractory (DU-145 and PC-3) and hormone-sensitive (LNCaP) prostate cancer cell lines was isolated by the SV RNA Isolation System (Promega, Madison, WI). Five hundred ng of total RNA were used for the first-strand cDNA synthesis using SuperscriptII RNase H^- Reverse Transcriptase (Life Technologies, Inc. Rockville, MD). cDNA fragments of the OSMRβ and β2-microglobulin (internal control) were amplified using the following primers: OSMRβ sense (5′-CCT GAA AAC CAG AAC TGG CTC-3′), OSMRβ antisense (5′-GTC CTT GGG CTC CTC AAG TAC TTT-3′), β2-microglobulin sense (5′-ATG CCT GCC GTG TGA ACC ATG T-3′), and β2-microglobulin antisense (5′-AGA GCT ACC TGT GGA GCA ACC T-3′; GenXpress, Vienna, Austria). For the amplification of the OSMRβ, 4 μl of cDNA solution were added to 46 μl of PCR mix containing 10× buffer (Finnzyme, Vienna, Austria; 200 nM dNTPs, 0.5 μmol of the respective oligonucleotide, 2% DMSO, and 2 units of DynaZyme II DNA polymerase). The same protocol was followed for the amplification of β2-microglobulin cDNA, with the distinction that 2 μl of cDNA solution and DMSO, at a final concentration of 4%, were added. PCR was performed in the GeneAmp PCR 9700 System thermocycler (PE Biosystems, Norwalk, CT) according to the following programs: (a) for the amplification of the *OSMR*β gene: 3 min at 94°C; 33 cycles of 45 s at 94°C, 10 s at 96°C, 1 min and 5 s at 57°C, and 1 min and 20 s at 73°C; with a final extension time 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, and 1min and 15 s at 73°C; and a final extension for 2 min at 73°C. The 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.

Transfections and CAT Assays. Transient transfection experiments by lipofection in DU-145 and PC-3 cells used the reporter plasmid ARE_2TATA-CAT and AR cDNA and were performed as described previously (6). The dn STAT3 plasmid REF STAT3CYF was kindly provided by Dr. Thomas Welte (Department of Pathology, Yale University, New Haven, CT). Reporter gene activity was measured after a 24-h incubation with OSM and/or androgen in the absence or presence of antiandrogens or PK inhibitors by CAT assay.

Western Blot. AR expression was investigated in transfected DU-145 cells and in controls. Cells were lysed as described elsewhere (17), and electrophoresis and Western blotting were performed using the NuPAGE electrophoresis system (Invitrogen, Leek, the Netherlands). For AR expression, the mouse monoclonal anti-AR antibody F39.4.1 (Biogenex, San Ramon, CA) at a dilution 1:1000 and the secondary antimouse horseradish peroxidase-linked antibody (1:1000 dilution) were used. A mouse anti-actin monoclonal antibody from Chemicon (Harrow, United Kingdom) was used at a dilution 1:1000. Signal was detected by the enhanced chemiluminescence detection system (Amersham Pharmacia Biotech, Freiburg, Germany).

Statistics. The Mann-Whitney *t* test was used for assessment of statistical significance.

RESULTS

Expression of the OSMRβ in Prostate Cancer Cell Lines. Before performing transfections of the androgen-responsive reporter gene, expression of the specific receptor OSMRβ was investigated in the prostate cancer cell lines LNCaP, PC-3, and DU-145 by RT-PCR. For OSMRβ expression studies, RT-PCR has been chosen rather than Western analysis because previous reports showed that commercially available antibodies could not be used for immunoblotting (18). The 743-bp OSMRβ fragment could be detected in all three of the cell lines. However, OSMRβ expression was much lower in the androgen-sensitive cell line LNCaP than in DU-145 or PC-3 cells (Fig. 1).

Activation of the AR by OSM in the Absence or Presence of Nonsteroidal Antiandrogens. To overcome difficulties that might occur because of the reduced OSMRβ expression in LNCaP cells, transfections were carried out in DU-145 and PC-3 cells with wild-type AR cDNA. Treatment of transfected DU-145 cells with OSM resulted in ligand-independent and

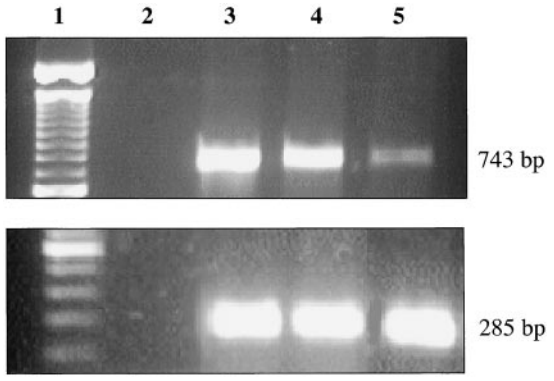

Fig. 1 Expression of OSMRβ fragments in prostate cancer cell lines. Total RNA was isolated from DU-145, PC-3, and LNCaP cells. cDNA fragments of OSMRβ (*top panel*) and β2-microglobulin (internal control, *bottom panel*) were amplified by RT-PCR; *Lane 1*, DNA size markers; *Lane 2*, negative control; *Lane 3*, DU-145 cells; *Lane 4*, PC-3 cells; *Lane 5*, LNCaP cells.

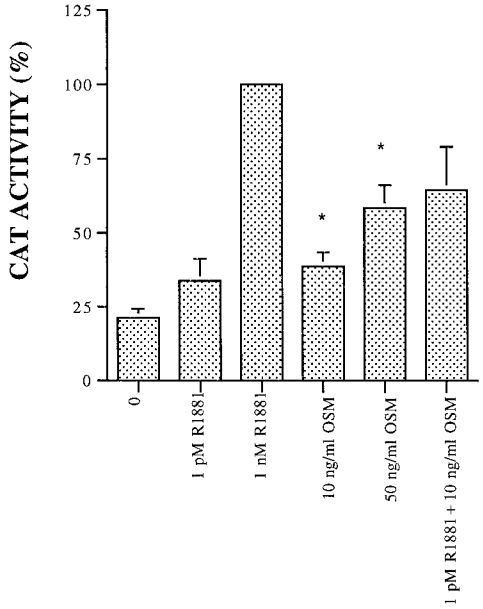

Fig. 2 AR-mediated reporter gene activity induced by OSM. DU-145 cells were transiently transfected with the androgen-responsive reporter gene *ARE$_2$TATA-CAT* and treated with R1881 and/or OSM for 24 h. Reporter gene activity was measured in cell extracts. CAT activity measured in cells treated with 1 nM R1881 was set as 100%, and all of the other activities are expressed in relation to that value. Four independent experiments were performed; *bars*, ±SE (*, $P < 0.05$; OSM treatment *versus* untreated control; the Mann-Whitney *t* test).

dose-dependent activation of the AR (Fig. 2). The maximal activation of the AR by OSM was 62% of the activity measured after treatment with the synthetic androgen R1881 (1 nM). Low doses of R1881 and OSM up-regulated the transcriptional activity of the AR in an additive manner. In contrast to DU-145 cells, there was no induction of reporter gene activity by OSM in the PC-3 cell line (data not shown). In previous publications, antiandrogens were shown to inhibit AR-mediated reporter gene

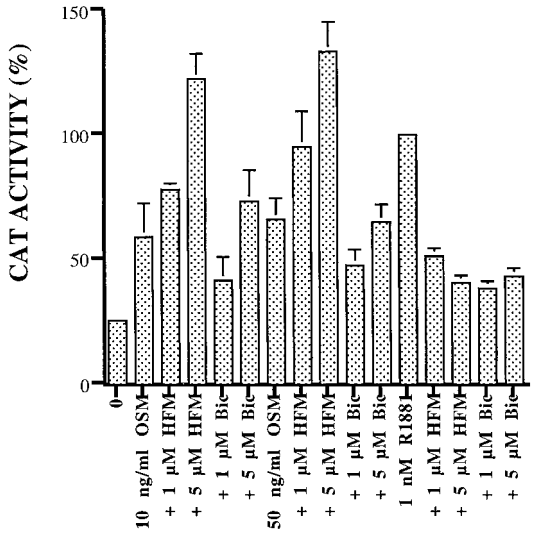

Fig. 3 Inhibition of OSM- and R1881-induced AR activity by the nonsteroidal antiandrogens hydroxyflutamide (*HFM*) and bicalutamide (*Bic*); four experiments. Transfection procedure was the same as that described in the legend for Fig. 2; *bars*, ±SE.

activity induced by the related cytokine IL-6 (6, 8). To study the ability of clinically used antiandrogens to block OSM-induced activation of the AR, DU-145 cells were incubated with OSM in the absence or presence of either hydroxyflutamide or bicalutamide. Surprisingly, hydroxyflutamide enhanced the OSM-induced AR activation, whereas bicalutamide down-regulated the ligand-independent activation, only when administered at a concentration of 1 μM (Fig. 3). When bicalutamide was added at a concentration of 5 μM, OSM-induced AR activity either slightly increased (with 10 ng/ml OSM) or remained unchanged (with 50 ng/ml OSM; Fig. 3). As expected, both AR antagonists suppressed AR activity caused by R1881 (Fig. 3). Thus, action of commonly used antiandrogens substantially differs in the presence of IL-6 or OSM.

Effect of PK Inhibitors on AR Activation. Previous studies revealed that ligand-independent activation of the AR depends on the functionality of various kinase pathways (3, 6). To investigate the involvement of the PKA pathway in the up-regulation of activity of the AR by OSM, the cells were treated with the PKA inhibitor PKI. Interestingly, PKI had no influence on AR activation exerted by OSM, whereas it down-regulated the db cAMP effect on AR activation (Fig. 4). Similar to those observations, the selective inhibitor of the extracellular signal-regulated kinase 1/2 kinases, PD 98059, caused only a minor (less than 25%) inhibition of OSM-induced reporter gene activity (Fig. 5). Control experiments confirmed that AR activation by EGF could be diminished by PD 98059. In addition, administration of dn STAT3 did not counteract AR activation by OSM (data not shown).

Expression of AR in OSM-treated DU-145 Cells. Nonsteroidal activators of the AR might also up-regulate receptor expression, as demonstrated with IL-6 in LNCaP cells (8, 19). To investigate a possible effect of OSM on AR protein levels, Western blots for the detection of the AR were performed. AR

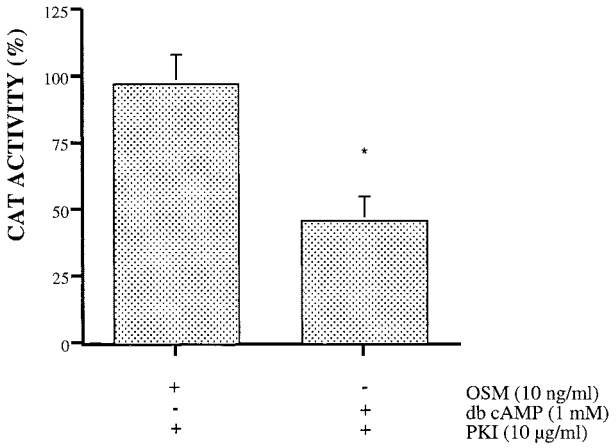

Fig. 4 Regulation of AR activity by the inhibitor of the PKA pathway, PKI. DU-145 cells were pretreated with PKI for 2 h before incubation with either OSM or the cAMP analogue db cAMP. CAT activities induced by OSM and db cAMP were set as 100%, respectively. The results are mean values of three independent experiments; *bars*, ±SE (*, $P < 0.05$; inhibition of OSM-induced *versus* inhibition of db cAMP-induced CAT activity; the Mann-Whitney *t* test).

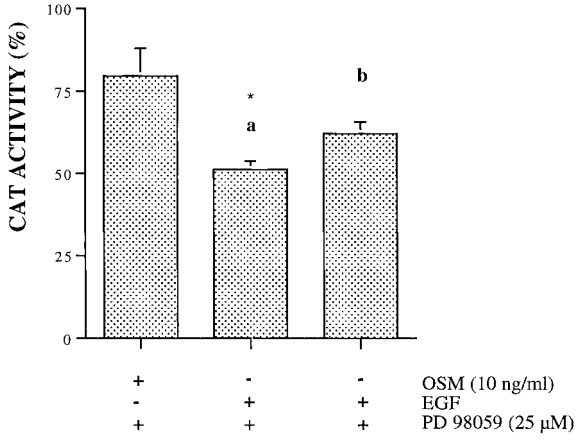

Fig. 5 The effect of the MAPK kinase inhibitor PD 98059 on AR activity. After pretreatment with PD 98059, DU-145 cells were incubated in the presence of either OSM or EGF (a, 10 ng/ml; b, 25 ng/ml). CAT activities measured in cells treated with OSM, 10, and 25 ng/ml EGF were set as 100%, respectively. CAT activity was measured in three experiments; bars, ±SE (*, $P < 0.05$; inhibition of OSM-induced *versus* inhibition of EGF-induced CAT activity; Mann-Whitney *t* test).

expression was analyzed in nontransfected, transfected untreated, and OSM-treated DU-145 cells. As expected, the AR was not detected in nontransfected DU-145 cells. AR expression was identical in transfected untreated and OSM-treated cells (Fig. 6). Thus, the increase in reporter gene activity caused by OSM is solely attributable to an increase in AR activity and not to up-regulation of AR expression.

DISCUSSION

OSM, which is a member of the IL-6 type of cytokines, affects the growth of prostate cells in a paracrine manner (16, 20). This is different from IL-6, for which both autocrine and paracrine loops were reported in the prostate (12, 19). It is well established that prostate cancer cells PC-3 and DU-145 express IL-6 (20–22). Those findings were supported by studies carried out on clinical specimens (12, 13). In contrast, OSM was not detected in conditioned media from the cell lines LNCaP ATCC, LNCaP GW that acquires a p53 mutation, PC-3, and DU-145 by ELISA (20, 21). On the basis of our results and those of previous reports, we conclude that both IL-6 and OSM activate the AR (6–8). However, the unexpected findings of the present study are that hydroxyflutamide acts as an AR agonist in the presence of OSM and that bicalutamide causes inhibition of AR activity only at lower concentrations. In prostate cancer, the switch of nonsteroidal antiandrogens to agonists was reported in various clinical situations and in *in vitro* and *in vivo* models: a) mutant AR are commonly activated by hydroxyflutamide (23, 24); (b) bicalutamide becomes an agonist after either prolonged steroid depletion or treatment with tumor necrosis factor-α (25, 26); and (c) reduced efficacy of AR antagonists was reported in cases of receptor activation by HER-2/neu or phorbol ester (5, 27, 28). Each of these mechanisms may be, at least in part, responsible for the antiandrogen withdrawal syndrome, the characteristic features of which are a decline of serum prostate-specific antigen levels and an improvement of clinical symptoms after the cessation of antiandrogen administration (29).

Although the signaling of IL-6 and OSM is similar in many cell types, some differences in structural organization of their receptors and in the recruitment of adapter proteins occur. On activation, the IL-6 receptor is composed of two gp130 signal transducing subunits and an IL-6 receptor α subunit, whereas, in case of the OSMR, the α subunit is absent and one gp130 subunit couples to either OSMRβ or LIF receptor β. Recently, differences in signal transduction between gp130 and the OSMR were revealed: after receptor activation, the adapter protein Shc binds only to the OSMRβ (and not to gp130), whereas the tyrosine phosphatase SHP-2 binds to gp130 (and not to the OSMRβ; Ref. 30). Whereas SHP-2 provides a link to the Ras/Raf/MAPK pathway, Shc, which is recruited by OSM, can mediate both Ras-dependent and Ras-independent responses. Therefore, the stronger effects of OSM reported in various cell lines are not surprising.

We have confirmed the previous finding that the OSMRβ is expressed in the androgen-insensitive prostate cancer cell lines DU-145 and PC-3 (16). In a report by Mori *et al.* (16), the OSMRβ was not detectable in the LNCaP cell line. However, we have observed that expression of the OSMRβ receptor is weaker in LNCaP than in DU-145 or PC-3 cells. These variations may reflect subtle differences in cell culture conditions and RT-PCR procedures.

Another important difference between IL-6 and OSM is that with the latter cytokine, inhibitors of neither PK nor dn STAT3 substantially antagonize AR activation. These data could be interpreted in two ways: (*a*) that inhibition of a single transduction pathway is compensated by other signal transduction cascades; or (*b*) that the effect of OSM depends on stimulation of other kinases. It is well established that the functional activity of the AR can be stimulated in a ligand-independent manner by activators of the PKA pathway, like forskolin (3, 31,

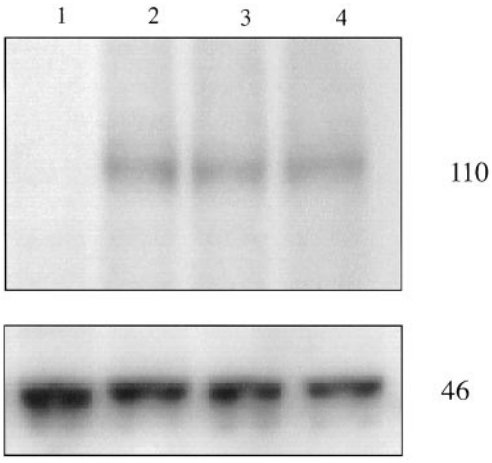

Fig. 6 Regulation of AR protein expression by OSM. Cell lysates were prepared from nontransfected and transfected DU-145 cells, and Western blots for the AR (*top panel*) and β-actin (internal control, *bottom panel*) were performed; *Lane 1*, nontransfected cells; *Lane 2*, DU-145 cells transfected with AR cDNA, no treatment; *Lane 3*, transfected cells treated with 10 ng/ml OSM; *Lane 4*, transfected cells treated with 50 ng/ml OSM.

32). In previous reports, administration of PKI, a specific PKA inhibitor, partially suppressed androgen-induced reporter gene activity and abolished the effects of nonsteroidal activators without changing AR expression (3, 6). PKI did not antagonize AR activation in the case of OSM, whereas it could reverse activation of the AR by the cAMP analogue. As mentioned previously, activation of the AR is also potentiated by MAPK (5). OSM effects on the increase of phosphorylated levels of MAPK extracellular signal-regulated kinase 1 and 2 were observed in a variety of cell lines (18, 33). We found that OSM-mediated activation of the AR could be only partially down-regulated by the MAPK kinase inhibitor PD 98059. Thus, we hypothesize that there must be other pathway(s) involved in AR activation through OSM. In PC-3 cells, which do not express STAT3 (34), OSM did not induce AR activity. This finding suggests that STAT3 signaling is required for AR activation in PC-3 cells. On the other hand, dn STAT3 did not antagonize OSM effect in DU-145 cells. Spiotto and Chung (34) demonstrated that STAT3 is present in DU-145 cells but, in comparison with the LNCaP cell line, its transcriptional activity is very low. On the basis of our results with the two cell lines, it could be proposed that there are differences in AR signaling between DU-145 and PC-3 cells transfected with AR cDNA. In glioblastoma cells and mammary tumors, OSM retards cellular proliferation, contrary to its effects in prostate cancer (18, 33, 35). Similar to our results on AR activation, neither the inhibitor of MAPK nor dn STAT3 affected the OSM regulation of the growth of glioblastoma cells (18). In breast cancer, a MAPK functional pathway is required for OSM-induced differentiation in ER-negative MDA MB231 but not in estrogen-sensitive MCF-7 cells (33).

Our finding that AR protein expression is not changed in cells transfected with AR cDNA and treated with a nonsteroidal activator is in agreement with previous studies (3). Nazareth and Weigel (3) showed that, in CV-1 and PC-3 cells, forskolin activated AR function without changing receptor levels.

In summary, agonistic behavior of AR antagonists is a particularly interesting aspect of AR activation by OSM. Acquisition of agonistic properties of nonsteroidal antiandrogens in tumor cells treated with OSM may compromise their use in prostate cancer treatment. In this context, there are two possibilities for therapeutic intervention; therapy concepts aimed to interfere with signaling of the IL-6 type of cytokines or to down-regulate AR expression should be worked out (17).

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