Osteoblast-Derived Factors Induce Androgen-Independent Proliferation and Expression of Prostate-Specific Antigen in Human Prostate Cancer Cells

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ABSTRACT

Purpose: Prostate cancer metastasizes to the skeleton to form osteoblastic lesions. Androgen ablation is the current treatment for metastatic prostate cancer. This therapy is palliative, and the disease will return in an androgen-independent form that is preceded by a rising titer of prostate-specific antigen (PSA). Here, we investigated the possibility that human osteoblasts might secrete factors that contribute to the emergence of androgen-independent prostate cancer.

Experimental Design: Primary cultures of human osteoblasts were used as a source of conditioned medium (OCM). Proliferation, expression of androgen-regulated genes, and transactivation of the androgen receptor (AR) were monitored in LNCaP human prostate cancer cells in response to OCM using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, Northern blot analysis, and reporter gene constructs. Levels of interleukin-6 (IL-6) present in OCM were measured, and its contribution to proliferation and expression of PSA were investigated by neutralization studies with anti-IL-6 antibodies.

Results: OCM increased the proliferation and expression of PSA at both the protein and RNA levels in LNCaP cells. Synergistic increases in the activities of PSA (6.1 kb)- and pARR3- tk-luciferase reporters were measured in cells cotreated with both OCM and androgen. OCM targeted the NH2-terminal domain of the AR. The effect of OCM on transcriptional activity of the AR was inhibited by an anti-androgen. Neutralizing antibodies to IL-6 blocked proliferation and expression of PSA by OCM.

Conclusion: Osteoblasts secrete factors, such as IL-6, that cause androgen-independent induction of PSA gene expression and proliferation of prostate cancer cells by a mechanism that partially relies on the AR. Identifying such molecular mechanisms may lead to improved clinical management of metastatic prostate cancer.

INTRODUCTION

Localized carcinoma of the prostate (CaP) can potentially be cured by surgery or radiation therapy. However, the only treatment available for advanced metastatic disease is the withdrawal of androgens, which are essential for the survival of prostate epithelial cells (for review, see Ref. 1). Clinical response to androgen ablation therapy is temporary with the ultimate development of androgen-independent disease that is preceded by an increasing titer of serum prostate-specific antigen (PSA). Unlike most malignancies, up to 85% of CaP metastases occur in the bone as osteoblastic (bone forming) lesions. Osteoblastic lesions are the major cause of CaP-related morbidity, and mortality and even small, bone-limited tumor burden in patients are strongly correlated to cachexia and death (for review, see Ref. 2).

Bone is mostly composed of an acellular collagen matrix and abundant in immobilized growth factors. Dispersed throughout this matrix are osteoblasts and osteoclasts, which are the cells responsible for bone maintenance. In bone, remodeling osteoclasts degrade the matrix to release growth factors which stimulate osteoblasts to lay down new bone (for review, see Refs. 3 and 4). Many of these growth factors increase proliferation of prostate cancer cells. In vitro studies have shown increased proliferation of prostate cancer cells stimulated by conditioned media from or cocultured with osteoblasts, growth factor extracts, and individual growth factors derived from bone, such as interleukin-6 (IL-6), insulin-like growth factor I (IGF-I) and IGF-II, epidermal growth factor, keratinocyte growth factor, bone morphogenic proteins, fibroblast growth factors, transforming growth factor β, cytokines, platelet-derived growth factor, vascular endothelial growth factor, and endothelin-1 (5–13). Many of these growth factors may be involved in circumventing the need for androgen in advanced disease by a mechanism involving ligand-independent activation of the androgen receptor (AR; Refs. 6 and 14–17).

The AR is a transcription factor that binds androgens and regulates gene expression required for normal male sexual development and maintenance of secondary sex characteristics (for review, see Ref. 1). It is expressed in the majority of prostate cancer tissue specimens, including androgen independ-
ent or hormone refractory disease, and is therefore a strong candidate for mediating androgen resistance (18–20). The AR is composed of three domains. Centrally located is the DNA-binding domain (DBD) that binds to androgen response elements (AREs) in upstream regulatory regions of androgen-regulated genes, such as PSA. The most COOH-terminal region comprises the ligand-binding domain that binds androgens and antiandrogens, such as bicalutamide. The ligand-binding domain contains a weak activation function-2 region and is separated from the DBD by a hinge region, which mediates nuclear localization. The NH2-terminal domain (NTD) is the most variable in sequence homology between species and contains the activation function-1 region required for transactivation (for review, see Ref. 1). The AR can be activated in an androgen-independent manner by a number of factors, including IL-6, IGF I, IGF II, keratinocyte growth factor, epidermal growth factor, forskolin, and cAMP, and some factors such as IL-6 and cAMP have been shown to mediate activation via the AR NTD (6, 14–16). Because the bone environment is rich in many of these growth factors, it has been suggested that bone-derived factors may facilitate survival and progression of CaP to androgen independence by cross-talk with the AR and alternative signal transduction pathways (5, 8, 21).

Clinical and experimental evidence suggests that one of the more important factors in prostate cancer progression to androgen independence and development of osseous metastases is IL-6. Elevated levels of IL-6 have been found in sera from patients who have metastatic and/or hormone refractory disease (22–26). IL-6 was first characterized as a modulator of an immune response that is produced by T cells and involved in hematopoiesis and inflammation (for review, see Ref. 27). However, IL-6 has been subsequently shown to be produced by many other cell types, including normal and malignant prostate epithelial cells and bone cells (28–34). It is clear that IL-6 has the potential to act in both a paracrine and autocrine manner. Importantly, IL-6 has been shown to enhance proliferation of prostate cancer cells, including LNCaP (6, 28), and induce transcription of androgen-regulated genes, such as PSA, by a mechanism of ligand-independent activation of the AR (6, 16). Thus, IL-6 derived from either bone or cancer cells may initiate and/or sustain progression of CaP to androgen independence. Here, we examined the effects of osteoblast-derived factors on the proliferation, expression of PSA, and ligand-independent activation of the AR in prostate cancer cells and confirm that IL-6 secreted from osteoblasts may be an important factor underlying androgen-independent disease.

MATERIALS AND METHODS

Cell Culture and Materials. LNCaP human prostate cancer cells between passage 37 and 55 were maintained in RPMI 1640 supplemented with 5% volume for volume fetal bovine serum (FBS; Invitrogen), 100 units/ml penicillin, and 100 μg/ml streptomycin (Invitrogen) and incubated at 37°C in an atmosphere of 5% CO2. All other chemicals were purchased from Sigma unless otherwise stated. Human recombinant IL-6 and antibodies to human IL-6, the IL-6 receptor, and IL-1β used in neutralization studies were purchased from R&D Systems. All experiments were performed using serum- and phenol red-free conditions.

Culturing of Osteoblast-Like Cells and Preparing Osteoblast-Conditioned Medium (OCM)-MEM. Human osteoblast-like cells were cultured from femoral head trabecular explants obtained from osteoarthrits patients undergoing hip/knee replacement surgery. To minimize possible patient variation, donors were restricted to males < 65 years of age. Trabecular bone was scraped into bone chips and further processed with a mortar and pestle. Bone chips were cultured in MEM containing 20% FBS at 37°C in the presence of 5% CO2 (35). The outgrowth of the osteoblast-like cells was monitored visually under the microscope with the aid of Gram Safaran staining. Cells were characterized using the reverse transcriptase-PCR for expression of the following osteoblast markers: (a) type I pro-collagen; (b) alkaline phosphatase; and (c) osteocalcin (35). When the osteoblast-like cells were confluent, they were washed with PBS (3 × 20 ml) and cultured in 20 ml of serum-free MEM. After 48 h, the OCM was collected and centrifuged or filtered through 0.22-μm Nalgene units to remove the cellular debris before storing at −80°C until use. OCMs from primary cultures of osteoblasts prepared from different patients were never pooled but rather used individually. Primary cultures of osteoblasts were grown and maintained in Falcon Primaria T75 flasks, with a surface of 75 cm2. The average protein concentration in OCM was 0.28 ± 0.05 mg/ml (SE). Fibroblast-conditioned media were collected and used as reported previously (5).

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium Bromide (MTT) Assay. LNCaP cells (1 × 104) were plated in 96-well Falcon tissue culture plates in RPMI containing 0.5% FBS in a final volume of 0.1 ml. When the cells reached 60% confluence, usually within 24 h, they were treated with R1881 or OCM collected from preparations of osteoblasts from three individual patients. After 5 days of culture, cell proliferation was assessed by adding 50 μl of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide dye (1 mg/ml) in serum-free media to the cells. After 4 h of incubation, the cells were solubilized in Me2SO (150 μl/well) on a shaker at room temperature before reading the absorbance at 570 nm using a Dynex Technologies Microplate Reader (6, 14).

PSA Protein and mRNA Expression. LNCaP cells were seeded at 1.5 × 106 cells/10 cm2 or 4 × 106 cells/15 cm2 dish in RPMI 1640 containing 5% FBS and cultured for 48 h before incubation in 10 or 20 ml of serum- and phenol red-free RPMI 1640 for an additional 48 h. This medium was removed, and the cells were pretreated with 5 or 10 ml of fresh RPMI for 2 h before adding an equal volume of treatment medium containing either OCM in MEM, 0.2 μl R1881, or both for a final concentration of 50% OCM and 0.1 μl R1881. After incubation for an additional 48 h, total RNA was extracted from LNCaP cells with TRIzol (Invitrogen), and 15 μg of RNA were electrophoresed on a 1% denaturing agarose gel before transfer to Hybond-n + filters (Amersham Pharmacia Biotech) by capillary diffusion. Northern blots were probed with a α32P-labeled (Amersham-radioactive probe and Invitogen-random labeling kit) 1.4-kb EcoRI PSA cDNA probe, quantitated using a STORM 860 Phosphorimager (Molecular Dynamics), and normalized to glyceraldehyde-3-phosphate dehydrogenase, which
was probed with a $\alpha^{32}$P-labeled, 1-kb BamHI glyceraldehyde-3-phosphate dehydrogenase fragment (36). Quantitation of secreted PSA protein by LNCaP cells was performed using $1 \times 10^7$ cells/well seeded in 12-well plates and treated as described above for the Northern blot experiment. Cells were cultured in 1 ml of medium for 48 h and serum starved in 0.6 ml of RPMI for an additional 48 h before an equal volume of treatment media was added. Supernatants from treated cultures were collected after 72 h of incubation. Total cellular DNA was extracted using the DNeasy kit according to the manufacturer’s protocol (Qiagen), and DNA was quantitated by absorbance at 260 nm. PSA protein in the culture medium (supernatant) was quantitated using the IMX total PSA kit (Abbott Laboratories) and normalized to total cellular DNA.

**Plasmids.** The pARR $_1$-tk-luciferase reporter contains three tandem repeats of the rat probasin AREs (−244 to −96) upstream of a minimal thymine kinase promoter in the pT81 vector (American Tissue Cell Collection; Ref. 37). The PSA (6.1 kb)-luciferase reporter contains the 6.1-kb promoter/enhancer region of the PSA gene and was kindly provided by Dr. J.-H. Hsieh (Department of Urology, South Western Medical School, Dallas, TX). The AR$_{1}$-ss8-Gal4DBD, Gal4DBD, and p5xGal4US-TATA-luciferase have been described previously (6, 14, 15).

**Transfection and Luciferase Assays.** Transfected cells were carried out using Lipofectin (Invitrogen) as described previously (6, 15). Briefly, LNCaP cells were seeded at $1 \times 10^5$ cells/well in 12-well NUNC tissue culture plates and cultured in RPMI 1640 supplemented with 5% FBS for 48 h. Cells were starved in RPMI for 24 h before transfection with 0.5 µg of PSA (6.1 kb)-luciferase or 0.5 µg of pARR$_1$-tk-luciferase and 1 µg of pGL2 basic vector (Promega) per well (to normalize the amount of DNA/well to 1.5 µg) using 2.5 µl/well Lipofectin in 0.6 ml of serum-free RPMI 1640. Transfection studies with the AR NTD were performed using 3 $\times 10^5$ cells seeded 24 h before cotransfecting with 5µg pGL4US-TATA-luciferase (1 µg/well) and AR$_{1}$-ss8Gal4DBD (50 ng/well) or Gal4DBD for an additional 24 h. The total amount of transfected plasmid DNA was normalized to 3 µg/well by the addition of empty vector with 5 µl of Lipofectin in 1 ml of serum-free RPMI 1640 using six-well Falcon tissue culture plates. After 24 h, the cells were supplemented with an equal volume of medium containing the appropriate treatment. For experiments using the PSA (6.1 kb) and pARR$_1$-tk reporters, OCM in MEM or MEM was added to 50% concentration with or without R1881 (0.1 µl or 10 nM), bicalutamide (20 µM), or 5 µg/ml (final concentration) of anti-IL-6, anti-IL-6R, or anti-IL-1β or both IL-6 antibody with bicalutamide and cultured for an additional 48 h. For AR$_{1}$-ss8-Gal4DBD transactivation and titration studies, forskolin, M$_{2}$SO (vehicle control for forskolin), human recombinant IL-6, R1881, or OCM was added and cultured for an additional 24 h. Harvested cells were resuspended in 1.5 ml of PBS containing 1 mM EDTA, pelleted by centrifugation, solubilized in passive lysis buffer, and assayed for luciferase activity using the Dual Luciferase Assay System according to the manufacturer’s protocol (Promega) with the EG&G Berthold multipeptide luminometer. Luciferase activity was normalized to protein concentration, which was determined by the Bradford assay (Bio-Rad) using γ-globulin as the standard (38). Each assay was done in triplicate, and experiments were repeated at least three times. Fold-induction represents the luciferase activity in the cells cultured in the treatment medium relative to that cultured in growth medium alone or vehicle control.

**IL-6 ELISA.** Anti-IL-6 monoclonal antibody (285 ng from R&D Systems) was immobilized on a 96-well plate for 2 h, and the wells were blocked with 1% BSA in PBS containing 0.01 M phosphate buffer (pH 7.4), 136 mM NaCl, and 2.7 mM KCl. Human recombinant (R&D Systems) or osteoblast-derived IL-6 was captured by the monoclonal antibody and then detected with a 1:2500 diluted polyclonal antibody (Sigma). Goat antirabbit immunoglobulin conjugated to horseradish peroxidase (1:5000) was used to label the bound polyclonal antibody, and o-phenylenediamine was used as the substrate in 0.1 M citrate-phosphate buffer (pH 5.0). Recombinant human IL-6 from R&D Systems was used to generate a standard curve for IL-6. The colorimetric development was monitored spectrophotometrically at 450 nm using a MRX microplate reader (Dynex Technologies). Antibodies, IL-6 standards, or OCM was diluted in PBS containing 1% BSA and 0.05% Tween. Washes were carried out between incubations with PBS containing 0.05% Tween.

**IL-6 Neutralization Assays.** LNCaP cells were seeded at $2.5 \times 10^4$ cells/well (four wells per treatment) in 24-well tissue (Primaria Falcon) culture plates and cultured in RPMI 1640 supplemented with 5% FBS for 48 h, then starved in 0.5 ml/well RPMI for an additional 48 h. The cells were then supplemented with 0.5 ml of medium containing OCM (50% final concentration) in MEM, MEM, and R1881 (10 nM final concentration). Neutralizing experiments used 10 µgl/ml antibodies to IL-6 and IL-1β, which was added to filtered OCM and then incubated for 30 min with occasional shaking at 37°C in a 5% CO$_2$ incubator before addition to LNCaP cells for a final antibody concentration of 5 µg/ml. This concentration is in the range reported previously for neutralization studies in prostate cancer cells (28). According to manufacturer’s instructions, 0.2–0.5 µg/ml antibody should neutralize $\approx$7.5 ng/ml IL-6 in media. Cells were harvested after 24 h of treatment. Total RNA was isolated in 0.25 ml of TRIzol (Invitrogen) reagent per well and processed according to the manufacturer’s instructions. Semiquantitative reverse transcriptase-PCR for PSA was performed using total RNA (0.5 µg) as described previously (6). For proliferation studies, cells were harvested after 5 days of incubation and analyzed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenylyltriazolium bromide assay as described above.

**Statistical Analysis.** Student’s $t$ test was used for statistical analysis. The significance level was set at $P < 0.05$, indicated by a * above the data point.

**RESULTS**

**Osteoblast-Derived Factors Induce Proliferation of LNCaP Prostate Cancer Cells.** To investigate the mechanism underlying androgen-independent growth of prostate cancer cells in response to osteoblast-derived factors, we first established whether LNCaP cells proliferated more rapidly in response to conditioned medium collected from primary cultures of human osteoblast-like cells as described previously (7, 8, 13). OCM was collected from osteoblast-like cells prepared...
from three different bone donors. As shown in Fig. 1, LNCaP cells proliferated more rapidly in response to incubation with OCM (50% final concentration). A concentration of 50% OCM was determined previously to be optimal for proliferation assays in prostate cancer cells (5). Untreated LNCaP cells (control) did not proliferate over the 5-day experiment. Androgen-treated cells (R1881, positive control) increased in proliferation as expected and reported previously (6, 14, 15). This suggests that there are factors present in OCM that promote proliferation of prostate cancer cells. Consistent with previous findings (5), no proliferation was observed in response to conditioned media from fibroblasts (data not shown).

**Osteoblast-Derived Factors Induce Expression of PSA.** Increasing levels of serum PSA indicate increased tumor burden, and elevation in serum PSA over nadir levels precede clinical indication of recurrent, androgen-independent prostatic bone lesions (39–43). Thus, we addressed the question whether osteoblast-derived factors have an effect on expression of PSA in LNCaP cells maintained in vitro. To do this, LNCaP cells were serum starved before incubation with OCM (50% volume for volume final concentration). Protein levels of PSA that were secreted into the culture media in response to OCM are shown in Fig. 2A. The basal level of secreted PSA protein was ~20 ng of protein/µg cellular DNA. OCM induced a 2.9-fold increase in the secreted level of PSA protein over control when normalized to cellular DNA content. R1881 induced a 7.4-fold increase in the secreted level of PSA as compared with control. These values were consistent with changes in levels of PSA mRNA in response to OCM. PSA mRNA was elevated 2.4-fold ± 0.2 SE in LNCaP cells incubated with OCM based on five independent experiments (Fig. 2B).

**Induction of Androgen-Responsive Reporter Gene Constructs by OCM.** To determine whether the induction of PSA mRNA and secreted protein by OCM are regulated at the transcriptional level, we used several reporter gene constructs that contain functional AREs. LNCaP cells were transfected with a PSA (6.1 kb)-luciferase reporter which contains several well-characterized AREs (44, 45). Optimal concentrations of R1881 (10 nM) activated this promoter ~100-fold (Fig. 3A) over control, which was consistent with previous reports (6, 14, 15). OCM caused a 3-fold induction of the PSA (6.1 kb)-luciferase reporter. A mixture of R1881 (10 nM) and OCM induced a synergistic increase in reporter activity that was ~250-fold over the control value.

The ARR-3-tk-luciferase reporter was next tested to determine whether another androgen-responsive reporter gene construct could be induced by OCM. This reporter consists of three repeats of the ARE1 and ARE2 region (six AREs in total) of the probasin gene ligated in tandem with the minimal thymidine kinase promoter in a luciferase reporter, thus making it highly sensitive to androgen induction. OCM increased the basal level of luciferase activity by ~3-fold (Fig. 3B). A mixture of R1881 (10 nM) and OCM induced a synergistic increase in reporter activity that was ~250-fold over the control value.
sensitive to R1881 (46). R1881 induced activity of the ARR3 reporter >400-fold (Fig. 3B) in transfected LNCaP cells. OCM induced this reporter ~10-fold over control. Cotreatment of cells with R1881 and OCM resulted in a synergistic increase of pARR3-tk-luciferase activity >800-fold over control (Fig. 3B).

Variability in the induction of reporter gene constructs was observed between OCM obtained from osteoblasts prepared from different bone donors. Induction of PSA (6.1 kb)-luciferase activity by OCM from different bone donors ranged from 3- to 24-fold (Fig. 3C). Similarly, induction of activity of ARR3-tk-luciferase by OCM ranged from 8- to 23-fold (Fig. 3D). Despite the variability, induction of reporters was clearly observed using OCM from all preparations of osteoblasts.

Bicalutamide Blocks OCM Induction of Androgen-Responsive Reporter Gene Constructs by OCM. Induction of both PSA (6.1 kb)- and pARR3-tk-luciferase reporters by OCM suggests the involvement of the AR because both of these reporters contain AREs. To further investigate the involvement of the AR, bicalutamide, a nonsteroidal antagonist of the AR, was used in combination with either OCM or R1881. As expected, bicalutamide blocked the induction of PSA (6.1 kb)-luciferase and pARR3-tk-luciferase reporter activity by R1881 treatment from ~60- to 6- and 38- to 4-fold, respectively (Fig. 4, A and B). The inhibition of OCM induction of these reporters was less, but remained statistically significant, i.e., from ~3- to 1.5- and 30- to 5-fold, respectively (Fig. 4, A and B). Together, our results indicate that bicalutamide is not as effective in blocking the induction of androgen-responsive reporters by osteoblast-derived factors when compared with blocking the effects of androgen.

OCM Activates the Human AR NTD. It has been previously shown that other compounds, such as forskolin, that activate the protein kinase A signaling pathway and IL-6 increase expression of PSA by targeting the AR NTD (6, 14, 15). Therefore, transactivation studies were used to examine whether OCM also activated the AR NTD. To do this, LNCaP cells were transfected with plasmids coding for either the AR1_558Gal4DBD fusion protein or Gal4DBD and the reporter construct that contains the Gal4DBD-binding element as described previously (6). Forskolin (50 μM) was included as a positive control, and it stimulated luciferase activity >5-fold (Fig. 5), which was consistent with previous reports using this optimal concentration (6, 15). A 4-fold stimulation
of the reporter was observed with OCM, demonstrating that OCM transactivates the AR.

Levels of IL-6 in OCM and Activation of the AR NTD by Recombinant Human IL-6. The AR NTD can be activated by IL-6 (6, 14), and osteoblasts secrete IL-6 (33, 47). It is therefore possible that the observed effects of OCM on PSA gene expression and activation of the AR NTD might be attributed to IL-6. Levels of IL-6 in OCM obtained from preparations of primary osteoblasts from four different patients were quantified by ELISA and shown to range from 1.69 to 3.4 ng/ml (Table 1), which correlates to activating levels reported in literature (6, 25, 26, 28, 33). This range of concentrations of IL-6 has been shown to be sufficient to activate the AR NTD (6, 14). Results in Fig. 6 show a 6-fold increase in luciferase activity obtained using 1 ng/ml recombinant IL-6, whereas a 13-fold induction was achieved with 10 ng/ml IL-6. R1881 was included as a negative control because androgen binds to the ligand-binding domain, which was not present in the AR1–558 Gal4DBD chimera. Together, these results strongly suggest that IL-6 at levels present in OCM is sufficient to transactivate the AR NTD.

Antibodies to IL-6 Block the Induction of PSA Gene Expression by OCM. OCM contains levels of IL-6 that are sufficient to cause transactivation of the AR NTD and induction of PSA promoter activity (Fig. 6; Ref. 14). Therefore, neutralizing antibodies to IL-6 and IL-6 receptor were applied to confirm the role of IL-6 in the mechanism of induction of PSA gene expression by OCM. As shown in Fig. 7A, induction of PSA (6.1 kb)-luciferase activity with OCM was not affected by an antibody to IL-1β. However, addition of antibodies to IL-6 or its receptor resulted in attenuated induction of PSA (6.1 kb)-luciferase activity by OCM from ~3-fold to slightly >1-fold. A combination of bicalutamide and neutralizing antibodies to IL-6 did not result in significantly greater attenuation of induction of

<table>
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<th>Table 1</th>
<th>Levels of IL-6* in OCM measured by ELISA (n = 3)</th>
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<tr>
<td>Bone donor no.</td>
<td>IL-6 (ng/ml) ± SD</td>
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<tr>
<td>18</td>
<td>3.40 +/- 0.04</td>
</tr>
<tr>
<td>19</td>
<td>2.99 +/- 0.08</td>
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<tr>
<td>20</td>
<td>1.69 +/- 0.04</td>
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<tr>
<td>21</td>
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* IL, interleukin; OCM, osteoblast-conditioned medium.
Fig 6 Titrations of recombinant interleukin (IL)-6 on transactivation of the androgen receptor NH2-terminal domain. Transactivation assays were performed in LNCaP cells and cotransfected with the 5xGal4UAS-TATA-luciferase (1 μg/well) and AR1 ss8Gal4DBD (50 ng/well) or Gal4DBD for 24 h before incubation with R1881 (10 nM), IL-6 (1, 10, 50 ng/ml), or vehicle for an additional 24 h. The total amount of plasmid DNA transfected was normalized to 3 μg/well by the addition of empty vector. The error bars represent the mean ± SD of three independent experiments. *, significantly increased over the control values; P < 0.05.

PSA (6.1 kb)-luciferase activity by OCM from that achieved with IL-6 antibody alone.

To ensure that neutralization of IL-6 in OCM had an effect on endogenous expression of PSA, semiquantitative reverse transcriptase-PCR was used with total RNA isolated from cells. As expected, 10 nM R1881 (positive control) induced levels of PSA mRNA, as did OCM (Fig. 7B, Lanes 2 and 3). Neutralizing antibodies to IL-6 completely blocked induction of PSA mRNA by OCM (Lane 4), whereas neutralizing antibodies to IL-1β had no inhibitory effect (Lane 5). These results are consistent with the reporter gene assays and suggest that IL-6 present in OCM contributes to the induction of PSA gene expression.

Antibodies to IL-6 Reduce Proliferation of LNCaP Cells in Response to OCM. As shown in Fig. 1, the proliferation of LNCaP cells was stimulated by OCM (50% final concentration). To determine the role of IL-6 in the enhanced proliferation of LNCaP cells in response to OCM, neutralizing antibodies to IL-6 and -1β were used. Neutralization of IL-1β in OCM had no effect on the proliferation of LNCaP cells in response to OCM (Fig. 8, compare Lanes 3 with 5). However, neutralizing IL-6 in OCM significantly reduced proliferation as compared with OCM treatment alone (compare Lanes 3 with 4). These results suggest that IL-6 is involved in the proliferative response of LNCaP cells in response to OCM.

DISCUSSION

Prostate cancer has the propensity to metastasize to the bone and form osteoblastic lesions. Treatment for advanced metastatic disease is androgen ablation therapy, which causes a temporary reduction in tumor burden concomitant with a decrease in serum PSA. Unfortunately, prostatic bone lesions will begin to grow again in the absence of androgens to form androgen-independent disease (39–43). Androgen-independent disease is biochemically characterized before the onset of symptoms by a rising titer of serum PSA (48). Rising levels of IL-6 in the serum of prostate cancer patients have also been suggested to be a surrogate marker for androgen-independent disease (24). Here, we examined the molecular mechanisms that may underlie the proliferation and expression of PSA in

Fig 7 Anti-interleukin (IL)-6 antibodies block the induction of prostate-specific antigen (PSA; 6.1 kb)-luciferase activity by osteoblast-conditioned medium (OCM). A, LNCaP cells were transiently transfected with PSA (6.1 kb)-luciferase (0.5 μg/well) for 24 h and pretreated for 2 h with bicalutamide (20 μM) before the addition of OCM (50% volume for volume) for an additional 48 h. Neutralization studies were performed using OCM that was pretreated with antibodies for IL-1β, IL-6, and IL-6R (5 μg/ml final concentration) before the addition to LNCaP cells. Cells were harvested, and relative luciferase activity was determined and reported as fold-induction. The error bars represent the mean ± SD of three independent experiments. *, significantly different; P < 0.05 between OCM compared with the control and OCM + IL6-R antibody (Ab), OCM + IL6 Ab, and OCM + IL6 Ab + bicalutamide (BIC) compared with OCM. In B, LNCaP cells were serum starved for 48 h, then treated with OCM, R1881, or OCM treated with neutralizing antibodies to IL-6 and IL1-β antibodies (5 μg/ml final concentration). Cells were harvested after 24 h, total RNA was isolated, and semiquantitative reverse transcription-PCT for PSA was performed with 0.5 μg of total RNA. Fold-induction of PSA is with respect to the control levels and normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH). This is a representative experiment of n = 7.
androgen-deprived prostate cancer cells in response to factors secreted by osteoblasts and have made the following observations: (a) OCM increased the proliferation of LNCaP prostate cancer cells; (b) OCM induced PSA gene expression; (c) OCM transactivated the AR; (d) IL-6 was present in OCM at levels sufficient to transactivate the AR; and (e) neutralizing antibodies to IL-6 and its receptor attenuated the induction of PSA gene expression and blocked proliferation of LNCaP cells in response to OCM.

Localized CaP is generally slow growing; however, once the disease becomes androgen independent, particularly in the bone, it becomes more aggressive (2, 49). The effect of OCM on the growth of LNCaP cells (Fig. 1) is consistent with clinical observations and provides experimental evidence that factors secreted by bone stimulate the proliferation of prostate cancer in the absence of androgens. Thus, osteoblast-derived factors appear to be in place to substitute for androgens by promoting the growth of metastatic prostate cancer cells in bone.

The serum level of PSA is directly correlated to tumor burden (43, 50). Here, we show that in addition to the proliferative response observed in the LNCaP cells exposed to OCM, PSA gene expression was also elevated. Induction of the PSA reporter gene construct was consistent with the elevated endogenous levels of PSA mRNA and secreted protein in LNCaP cells exposed to OCM (Figs. 2–4). These data correlate to the clinical observations of recurrent, androgen-independent disease. Interestingly, PSA gene expression appeared to be additive or synergistic when the synthetic androgen R1881 and OCM were used together and mRNA or reporter activity was measured. Previous studies have observed synergistic increases in PSA gene expression in LNCaP cells in response to forskolin and IL-6, R1881 and IL-6, and R1881 and butyrate (6, 14, 51). Typically, additive or synergistic changes are interpreted to imply that multiple signaling pathways are being used.

The AR is expressed in the majority of metastatic and hormone refractory prostate cancer tissues (18–20), and genes "normally" regulated by androgens become re-expressed in androgen-independent prostate cancer cells (52). Here, we suggest a role for the AR in the up-regulation of PSA gene expression by OCM attributable to the following observations: (a) OCM induced the transcription of both PSA and ARR3 reporter gene constructs, and both of these reporters contain several well-characterized AREs (44, 45, 53); (b) OCM increased transactivation of the AR; and (c) the antiandrogen bicalutamide partially blocked the induction of PSA and ARR3 reporters by OCM. Together, these data imply that factors present in OCM may activate the AR to induce genes containing AREs. This is consistent with the fact that several factors secreted by bone, such as IGFs, keratinocyte growth factor, epidermal growth factor, and IL-6, have been shown previously to activate the AR (6, 14, 16, 17). The precise mechanism for how the AR, and especially its NTD, are activated in the absence of its cognate ligand by cross-talk with these alternative pathways has not been elucidated but may involve changes in the phosphorylation state of the AR or an interacting protein, such as SRC-1 (14, 15, 54). Indeed, it has been shown recently that mitogen-activated protein kinase is required for both ligand-dependent and -independent activation of the AR and phosphorylation of SRC-1 by mitogen-activated protein kinase at serine 1185 and threonine 1179 is required for optimal ligand-independent activation of the AR in LNCaP cells (6, 14). Our data showing that the antiandrogen bicalutamide only partially blocks the induction of these ARE-driven reporters by OCM may imply the involvement of additional mechanisms to the AR. Alternatively, antiandrogens that bind the ligand-binding domain may not be effective antagonists for AR that is activated via its NTD by nonandrogenic pathways.

IL-6 is important in normal bone turnover, is elevated in the serum of prostate cancer patients with androgen-independent disease, causes ligand-independent activation of the AR, induces PSA gene expression, increases proliferation of prostate cancer cells, and is secreted by the bone (6, 14, 16, 23, 33). Here, IL-6 was shown to be present in OCM at concentrations that were sufficient to activate the AR (Table 1; Fig. 6; Ref. 14). Consistent with the idea of IL-6 being one of the main bone-derived mediators in promoting osseous lesions, we have shown the following: (a) OCM induced PSA mRNA to similar levels reported using recombinant IL-6 (14, 16); (b) both OCM and IL-6 transactivate the AR NTD (Figs. 5 and 6; Ref. 14); (c) both OCM and IL-6 increase the proliferation of LNCaP cells (Fig. 1; Refs. 14 and 28); and (d) neutralization studies with antibodies to IL-6 and its receptor blocked the induction of PSA reporter activity, endogenous PSA mRNA, and proliferation, whereas an antibody to IL-1β did not show inhibition providing an indication of specificity. Together, these data support a role for IL-6 as an important component secreted by bone in mediating androgen-independent expression of PSA in prostate cancer cells.

In conclusion, these studies show that factors secreted by primary cultures of human osteoblast-like cells stimulate prolif-

![Fig. 8](image-url)
eration and induction of PSA gene expression and androgen-regulated reporter gene constructs in prostate cancer cells devoid of androgens. OCM transactivated the AR, suggesting that the AR may play a role in the progression of prostate cancer to androgen independence by a mechanism initiated by factors secreted from osteoblasts, one of which appears to be IL-6. Additional studies of factors such as IL-6 will provide insight into new alternative pathways of growth regulation that can supplant the requirement for androgens in prostate cancer and result in better methods to prevent or control androgen-independent disease.

ACKNOWLEDGMENTS

We thank Dr. Katie Meehan for providing a critical reading of this manuscript.

REFERENCES


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