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Osteoblasts induce prostate cancer proliferation and PSA expression through interleukin-6-mediated activation of the androgen receptor

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Abstract

Prostate cancer (CaP) metastases selectively develop in bone as opposed to other sites through unknown mechanisms. Interleukin-6 (IL-6) is considered to contribute to CaP progression and is produced at high levels in osteoblasts. We hypothesized that osteoblast-derived IL-6 in the bone microenvironment contributes to the fertile soil for CaP growth. Accordingly, human CaP cells, LNCaP, C4-2B and VCaP, were treated with conditioned medium (CM) collected from human osteoblast-like HOBIT cells grown in androgen-depleted medium. We found that CM induced proliferation, prostate-specific antigen (PSA) protein and mRNA expression in a dose-dependent manner in these cell lines as determined by ELISA and real-time PCR, respectively. CM also activated the PSA promoter in these cells. Both HOBIT and primary osteoblast (POB) cells produced high levels of IL-6 measured by bioassay. LNCaP, C4-2B and VCaP cells expressed IL-6, but at much lower levels then the HOBIT and POB and they also expressed the IL-6 receptor mRNA, indicating they can respond to IL-6. Anti-IL-6 antibody added to HOBIT or POB CM dose-dependently inhibited the CM-induced cell proliferation and PSA expression in these CaP cell lines. HOBIT CM induced nuclear translocation of the AR and this was inhibited by anti-IL-6 antibody. Additionally, the antiandrogen bicalutamide inhibited HOBIT CM-induced cell proliferation. These results demonstrate that osteoblasts promote CaP growth through IL-6-mediated activation of the AR. Furthermore, these data underscore the importance of cross-talk between tumor and the bone microenvironment in the development of CaP bone metastases.

Abbreviations: AR – androgen receptor; BIC – bicalutamide; CaP – prostate cancer; CS-FBS – charcoal-stripped fetal bovine serum; CM – conditioned media; DMEM – Dulbecco's modified Eagles Medium; ELISA – enzyme-linked immunostaining assay; FBS – fetal bovine serum; FGF – fibroblast growth factor; GFP – green fluorescent protein; IGF – insulin-like growth factor; IL-6 – interleukin-6; POB – huamn primary osteoblast; PSA – prostate-specific antigen; SDF – stromal derived factor

Introduction

Prostate cancer (CaP) often progresses from an androgen dependent cancer into an androgen independent cancer that favors metastasis to bone compared to other sites in greater than 90% of patients with prostate carcinoma [1–2]. However, mechanisms accounting for metastatic CaP cells' skeletal preference are poorly understood. In accordance with Paget's 'seed and soil' hypothesis [3], it is possible that factors in the bone microenvironment may provide a 'fertile soil' allowing the 'seed' of CaP cells to grow. There are many factors derived from the bone microenvironment that may promote establishment and progression of CaP cells in bone [reviewed in 4] and contribute to cross-talk between the CaP cells and the bone microenvironment [reviewed in 5].

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Evidence in support of the possibility that bone-derived factors contribute to the development of CaP metastases has been derived from studies in which conditioned media from bone cultures induced cancer cell growth of a variety of tumor cell lines [6–8]. Several specific bone-derived factors have been demonstrated to stimulate CaP cell growth *in vitro*, including insulin-like growth factor-1 and 2 (IGF-1 and IGF-2) [9–10], fibroblast growth factor-8 (FGF-8) [8], stromal-derived factor-1 (SDF-1) [11] and osteonectin [12–13]. However, the mechanisms through which these factors influence CaP cell proliferation have not been delineated.

One factor that is active on CaP cells and is produced at high levels by osteoblasts is interleukin-6 (IL-6) [14]. IL-6 is a key mediator of inflammation and immunologic reactions [15]. Elevated serum IL-6 levels are associated with clinical morbidity of CaP patients [16–17]. IL-6 directly affects benign and malignant CaP cell proliferation depending on the cell type and differentiation stage [18–

19]. IL-6 induces prostate-specific antigen (PSA) expression through ligand-independent activation of the androgen receptor (AR) [20–21] that is mediated by multiple signaling pathways including MAP kinases and Jak/Stats [22–23]. The observation that osteoblasts produce high levels of IL-6 in combination with the fact that IL-6 has many affects on CaP cells suggests that bone-derived IL-6 may promote development of CaP metastases. Accordingly, in this study we tested the hypothesis that IL-6 contributes to osteoblast-induced CaP proliferation and PSA expression *in vitro*.

Materials and methods

Cell culture and reagents

The human prostate cell lines, LNCaP and PC3 (American Type Tissue Collection, Manassas, Virginia), were maintained in RPMI-1640 supplemented with 10% FBS, 100 U/l penicillin G, 100 μg/ml streptomycin, and 2 mM L-glutamine. The human CaP cell line C4-2B (UroCor Inc., Oklahoma City, Oklahoma), derived from LNCaP cells after several passages through castrated nude mice [24], were maintained in T medium, which consisted of 80% Dulbecco's modified Eagles medium (DMEM, Life Technologies Inc., Grand Island, New York), 20% F12K (Irving Scientific, Santa Ana, California), 3 g/l $NaHCO_3$, 100U/l penicillin G, 100 μg/ml streptomycin, 5 μg/ml insulin, 13.6 pg/ml triiodothyronine, 5 μg/ml apo-transferrin, 0.25 μg/ml biotin, 25 μg/ml adenine, and were supplemented with 10% FBS. The human CaP cell line VCaP, derived from a vertebral metastatic lesion [25], was maintained in DMEM supplemented with 10% FBS, 100 U/l penicillin G, 100 μg/ml streptomycin, and 2 mM L-glutamine. Human osteoblast-like HOBIT cells (kindly provided by Dr. L Riggs, Mayo Foundation, Rochester, Minnesota) were grown in DMEM supplemented with 10% (v/v) fetal calf serum, 2 mM L-glutamine and penicillin/streptomycin. Human primary osteoblasts (POB) were obtained from femoral head trabecular explants derived from patients undergoing hip replacement surgery. Tissue procurement was approved by the University of Michigan Institutional Review Board. Briefly, the trabecular bone was scraped into bone chips and further processed with a bone grinder. Bone chips were cultured in MEM supplemented with 20% FBS. The presence of osteoblast cells was confirmed by RT-PCR for alkaline phosphatase and osteocalcin mRNA expression and histochemical staining for alkaline phosphatase using the Sigma Diagnostics Phosphatase kit (Sigma Diagnostic, Inc, St. Louis, Missouri). Cultures consisted of greater then 90% osteoblasts. All cells were grown at 37 °C in a humidified atmosphere containing 5% CO_2. Anti-human IL-6 antibody (CLB, Amsterdam, The Netherlands) is a mouse monoclonal (subtype IgG1) specific for human IL-6. Mouse IgG1 (Sigma, St. Louis, Missouri) was used as an isotype control antibody. Recombinant human IL-6 was purchased from R & D systems (Minneapolis, Minnesota). The antiandrogen, bicalutamide (BIC) (Zeneca Pharmaceuticals, Macclesfield, UK) was provided by Dr. K. Olsen (University of Michigan, Ann Arbor, Michigan).

Preparation of conditioned media (CM) from HOBIT and POB

HOBIT and POB cells were grown in 10-cm tissue culture dishes with their respective maintenance medium until they reach 80% confluent, then the medium was replaced with 10 ml of DMEM with 0.5% charcoal-stripped FBS and continued culturing for 48 h. Supernatants were collected as CM. To normalize for differences in cell density due to proliferation during the culture period, cells from each plate were collected, and total DNA content/plate was determined (spectrophotometric absorbance at 260 nm). CM was then normalized for DNA content between samples by adding DMEM.

Cell proliferation assay

Cell proliferation was measured using the CellTiter 96 AQ nonradioactive cell proliferation assay (Promega Corp. Madision, Wisconsin). Briefly, LNCaP, C4-2B, and VCaP cells were plated in their maintenance medium in wells of a 96-well plate at 5,000/well in triplicates. After 12 h of culture, the medium was changed to DMEM plus 0.5% CS-FBS and a different concentration (0–50%) of CM and/or indicated concentration of anti-IL-6 antibody or isotype control (500 ng/ml), recombinant human IL-6 (10 ng/ml), or indicated concentration of anti-androgen, bicalutamide (BIC) was added. The cells were incubated for 48 h, and then 20 μl/well of combined MTS/PMS solution was added. After 1 h at 37 °C in a humidified 5% CO_2 atmosphere, the absorbance at 490 nm was recorded by using an ELISA plate reader.

Transfection and prostate specific antigen (PSA) promoter activity measurement

A 5.8 kb fragment of the human PSA promoter was subcloned into pGL-2 (Promega Corp, Madison, Wisconsin) to create pGLPSAp5.8. LNCaP, C4-2B, and VCaP cells were grown to 80% confluence in T75 flasks, trypsinized, washed in phosphate buffered saline (PBS), and replated at 1×10^5 cells/well in 12-well culture plate in maintaining media. Twelve hours after plating, cells were co-transfected with pGLPSAp5.8 (1 μg) and pRL-SV40 vector (Promega Corp; 50 ng) using Transfast transfection assay (Promega Corp). Twenty-four hours after transfection, the medium was changed to DMEM with 0.5% CS-FBS, and cells were treated with various concentrations (v/v) of conditioned medium (CM) collected from HOBIT or POB for 24 h. The cells were then lysed with luciferase lysis buffer (Promega Corp) and the luciferase activity was quantified using a luminometer (Turner Designs, Sunnyvale, California) using the protocol from Dual-Luciferase Reporter assay system (Promega Corp).

Measurement of PSA protein levels

LNCaP, C4-2B, and VCaP cells were plated in 12-well-plates in their maintenance medium at 3×10^5/well in triplicates. After 12 h of culture, the medium was changed to DMEM plus 0.5% CS-FBS and a different concentration (0–50%) of CM and/or indicated concentration of anti-IL-6 antibody or isotype control (500 ng/ml), or recombinant human IL-6 (10 ng/ml) were added. PSA protein in the culture supernatant was measured following the manufacture's protocol by using the ACCUCYTE human PSA assay kit (Cytiimmune Sciences Inc. College Park, Massachusetts) and normalized to total cellular DNA that extracted from the cells using DNeasy kit (Qiagen, Valencia, California). The sensitivity of the assay is 0.488 ng/ml.

Quantification of PSA mRNA expression by real-time PCR

LNCaP, C4-2B, and VCaP cells were plated in their maintenance medium into 12-well-plates at 3×10^5/well in triplicates. After 12 h of culture, the medium was changed to DMEM plus 0.5% CS-FBS and a various concentrations (0–50%) of CM were added. The cells were grown for 48 hours and total RNA was isolated from the cell lysates using Trizol® (Invitrogen, Carlsbad, California). Message RNA expression was determined using real-time PCR on the LightCycler (Roche Diagnostics) for PSA and normalization to β2-microglobulin. The primer sequences used were: PSA sense, 5′-GGCAGGTGCTTGTAGCCTCTC-3′; PSA antisense, 5′-CACCCGAGCAGGTGCTTTTGC-3′; β2-microglobulin, sense, 5′-ATGCCTGCCGTGTGAACCATGT-3′; and β2-microglobulin antisense 5′-AGAGCTACCTGTGGAGCAA CCT-3′.

Measurement of IL-6 by ELISA

CM collected from HOBIT and POB were assayed for IL-6 using a human ELISA kit (R&D Systems) as recommended by the manufacturer. The minimum dose of IL-6 detectable is 0.7 pg/ml. For measuring IL-6 production by CaP cells, LNCaP, C4-2B, VCaP, and PC3 cells were plated in their maintenance medium into10-cm tissue culture dishes and incubated until they reached 80% confluency, then the media was replaced with 10 ml of DMEM with 0.5% charcoal-stripped FBS. The cell supernatants were collected after an additional 48 h of culture. To normalize for differences in cell density due to proliferation during the culture period, cells from each plate were collected, and total DNA content/plate was determined (spectrophotometric absorbance at 260 nm). CM was then normalized for DNA content between samples by adding DMEM.

Measurement of bioactive IL-6 using B9 Bioassay

Bioactive IL-6 was measured using the B9 cell bioassay as previously reported with minor modifications [26]. Briefly, B9 cells were maintained in RPMI 1640 supplemented with 10% FBS and 5ng/ml recombinant human IL-6 (Promega Corp). Cells at 2×10^3/well in triplicates were plated in 100ul of RPMI 1640 with 10% FBS in 96-well plate. Various concentration of CM (v/v) from HOBIT and/or anti-IL-6 antibody or isotype control (both at 500 ng/ml) were added into the wells in volume of 100 ul. Recombinant human IL-6 was used as a positive control. The cells were cultured for 48 h at 37 °C in 5% CO_2 incubator. Then 20 μl/well of combined MTS/PMS solution (Promega Corp) was added into each well. After incubation of 1 h at 37 °C in a humidified 5% CO_2 atmosphere, the absorbance at 490 nm was recorded by using an ELISA plate reader.

Measurement of IL-6 receptor (IL-6R) mRNA expression by RT-PCR

LNCaP, C4-2B, VCaP, and PC3 cells were plated in their respective maintenance medium into 60 mm plates at 2×10^6 cells/plate. The cells were allowed to grow for 12 hours, total RNA was then isolated using Trizol (Invitrogen). IL-6R mRNA expression was determined using reverse transcriptase-polymerase chain reaction (RT-PCR). The primer sequences used were: sense, 5′-CATTGCCATTGTTCTGAGGTTC-3′; antisense, 5′-AGTAGTCTGTATTGCTGATGTC-3′, resulting in a PCR product of 251 bp. The PC3 cell line was used as a positive control for IL-6R expression. RT-PCR was performed with 1 μg of total RNA using the Access RT-PCR system (Promega Corp), as directed by the manufacturer, in a thermal cycler (GeneAmp PCR system 9700; Perkin-Elmer Applied Biosystems, Foster City, California) under the following conditions: first-strand cDNA was synthesized at 48 °C for 45 min; then denatured at 94 °C for 2 min for the first cycle and at 15 sec for additional 40 cycles; annealing was performed at 55 °C for 30 sec; and extension at 72 °C for 60 sec. Final extension was at 72 °C for 5 min. The PCR products were subjected to electrophoresis on a 1.5% agarose gel, stained with ethidium bromide.

Detection of AR nuclear translocation

Fluorescent detection of nuclear AR translocation was performed as a modification of a procedure we have previously described [20]. Briefly, an expression plasmid consisting of AR fused to green fluorescent protein (GFP) (a gift from Dr M. Lu, Harvard University, Boston, Massachusetts) was stably-transfected into C4-2B cells. Sterile glass coverslips were placed in wells of 12-well plates. C4-2B cells were then added in their maintenance medium at 3×10^5 cells/well. After 12 hours of culture, the medium was changed to DMEM plus 0.5% CS-FBS and 25% HOBIT CM and/or increasing dose of anti-IL-6 antibody or isotype control (500 ng/ml), or recombinant human IL-6 (10 ng/ml). Following 48 hrs of culture, the coverslips were rinsed once in PBS and fixed with 4% paraformaldehyde in PBS pH 7.4 for 10 min. Paraformaldehyde was then neutralized with a 5 min exposure to 50 mM NH4Cl in PBS. The coverslips were then washed twice in PBS. Coverslips were incubated for 15 min with 1% BSA, NFDM, 0.3% Triton X-100 in PBS, then for 1 h in rabbit anti-AR (N-20) (Santa Cruz Biotech-

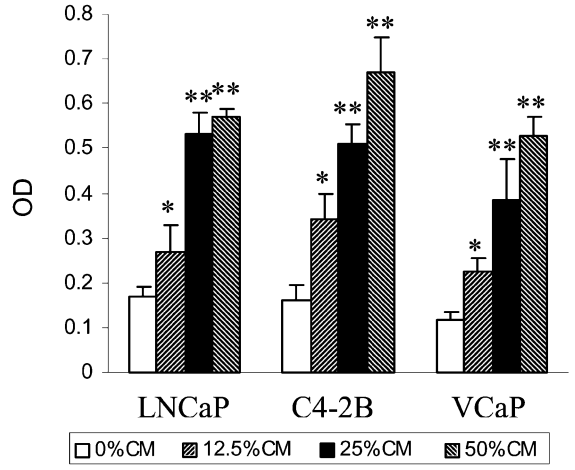

Figure 1. HOBIT CM induces LNCaP, C4-2B, and VCaP cell proliferation. LNCaP, C4-2B and VCaP cells were seeded in triplicates at 5000 cells/well in their maintaining media in 96-well plate. Twelve hours later, the media was changed to DMEM supplemented with 0.5% charcoal-striped FBS. Then LNCaP, C4-2B, and VCaP cells were treated various concentrations (v/v) of HOBIT CM as indicated. The cells were allowed to grow 48 hours, then the cell proliferation assay was performed using MTS assay. Data are presented as Mean ± SD of OD value. $^{*}P < 0.01$ vs 0% CM-treated cells; $^{**}P < 0.001$ vs 0% CM-treated cells.

nology, Santa Cruz, California) diluted 1:100 in 1% BSA, 5% NFDM, and 0.1% Tween 20 in PBS. The slips were then extensively washed in PBS-Tween-20 and incubated for 1 h with goat anti-rabbit IgG conjugated with FITC (Santa Cruz Biotechnology) diluted 1:500 in 1% BSA, 5% NFDM, and 0.1% Tween 20 in PBS. The images were captured using a fluorescent microscope.

Data analysis

One way ANOVA was used for all studies. Fisher's least-significant difference was used for post hoc analysis. *P*-values less than or equal to 0.05 were considered to be statistically significant.

Results

CM from HOBIT induces CaP cell proliferation

To test if the factors from human osteoblast-like HOBIT cells induce CaP cell growth, LNCaP, C4-2B, and VCaP cells were incubated with increasing dose of androgen-depleted CM collected from HOBIT cells. HOBIT CM induced proliferation in a dose dependent manner in these cell lines (Figure 1). When CaP cells were treated with 25% CM, the cell proliferation was induced over 2-fold compared to the no CM added controls. These results indicate that HOBIT cells produce soluble factors that induce CaP cell proliferation. This result is consistent with the previous report that LNCaP cell proliferation is increased in response to CM collected from primary human osteoblast culture [27].

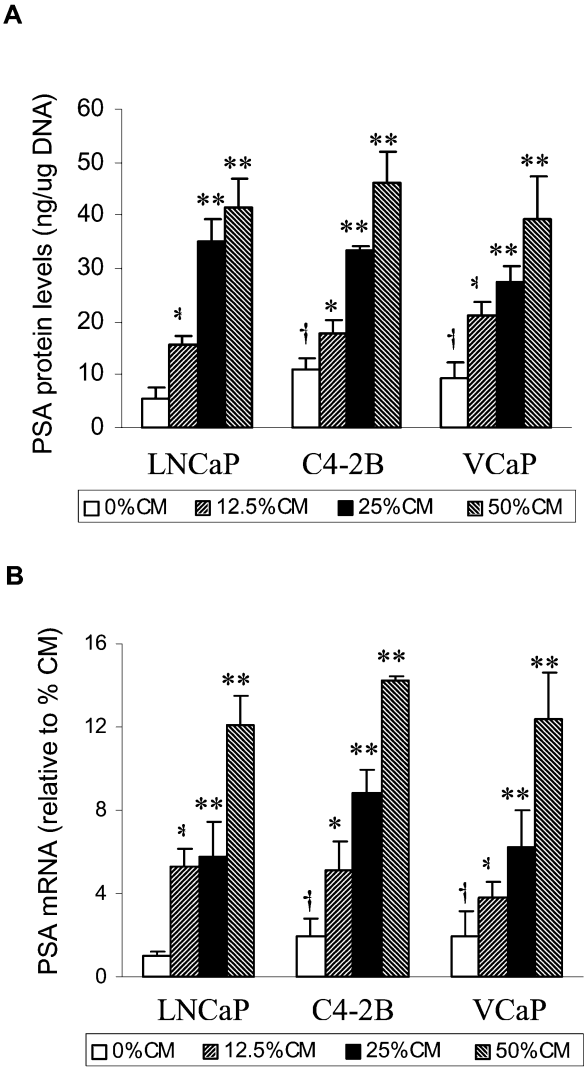

Figure 2. HOBIT CM induces PSA protein and mRNA expression in LNCaP, C4-2B, and VCaP cells. (A) LNCaP, C4-2B, and VCaP cells in their maintaining media were added to the 12-well-plate in triplicates. After 12 hours of culture, the media was changed to DMEM plus 0.5% CS-FBS and a different concentration (0–50%) of CM were added. PSA protein was measured using the ACCUCYTE human PSA assay kit and normalized to total cellular DNA that extracted from the cells using DNeasy kit. Data are presented as Mean ± SD from triplicates. $^{*}P < 0.01$ vs 0% CM-treated cells. $^{**}P < 0.001$ vs 0% CM-treated cells; $\dagger P < 0.01$ vs LNCaP cells. (B) Total RNA was isolated from the cell lysates. Message RNA expression was tested by real-time PCR for PSA and normalized to β2-microglobulin. Data are presented as Mean ± SD from triplicates. $^{*}P < 0.01$ vs 0% CM-treated cells; $^{**}P < 0.001$ vs 0% CM-treated cells; $\dagger P < 0.01$ vs LNCaP cells.

CM from HOBIT induces PSA protein and mRNA expression in CaP cells

To investigate if CM from HOBIT cells induce PSA protein and mRNA expression, LNCaP, C4-2B and VCaP cells were treated with increasing dose of androgen-depleted CM collected from HOBIT cells. HOBIT CM induced PSA protein and mRNA expression in LNCaP, C4-2B, and VCaP cells in a dose dependent manner (Figures 2A and B). When LNCaP, C4-2B, and VCaP cells were treated with 25% CM, the PSA protein levels were induced over 3-fold compared to the cells in which vehicle control CM was added control.

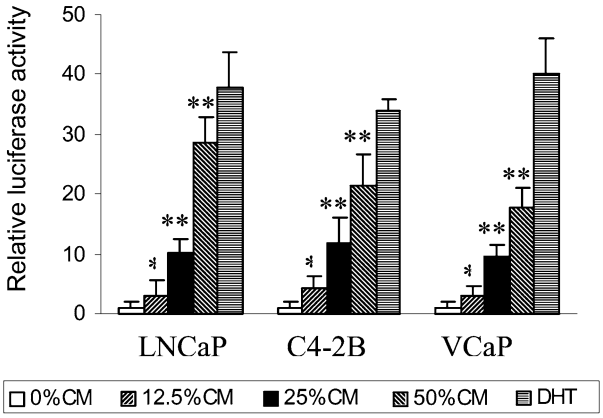

Figure 3. HOBIT CM induces PSA promoter activity in LNCaP, C4-2B, and VCaP cells. LNCaP, C4-2B and VCaP cells were transiently transfected with pGLPSAp5.8 and PRL-SV40 vector as internal control as described in Materials and methods. Cells were treated with various concentration of HOBIT CM or DHT (as positive control), cell extracts were assayed for luciferase activity. Data are represented as Mean ± SD of relative luciferase activity from triplicate samples. $^{*}P < 0.01$ vs 0% CM-treated cells; $^{**}P < 0.001$ vs 0% CM-treated cells.

The magnitude of induction of protein levels was consistent with the induction of PSA mRNA expression. These results suggest that HOBIT cells produce a soluble factor that has a pro-androgenic effect.

CM from HOBIT induces PSA promoter activity in CaP cells

To investigate whether the induction of PSA mRNA by HOBIT CM is due to induction of PSA gene transcription, we used a reporter plasmid containing the PSA promoter driving luciferase to transfect LNCaP, C4-2B, and VCaP cells. CM from HOBIT cells dose-dependently induced this promoter activity in these cells (Figure 3). CM at 25% induced the PSA promoter over 5-fold in these cell lines. This result indicates that HOBIT cell CM-induced PSA mRNA expression occurs through activation of PSA promoter transcription.

CM from HOBIT and primary osteoblasts (POB) contains bioactive IL-6

IL-6 has been considered to be an important factor in CaP progression. It has been shown that osteoblasts produce IL-6 [28] and IL-6 is able to activate the AR [20, 29]. Therefore, it is possible that IL-6 may contribute to HOBIT CM-induced PSA expression through activation of the AR. To test this, we first measured IL-6 levels in the HOBIT CM. HOBIT produced a high amount of immunoreactive IL-6 as determined by ELISA (Figure 4A). To test whether the secreted IL-6 was bioactive, the HOBIT CM was subjected to B9 bioassay for IL-6. HOBIT CM induced B9 cell proliferation in a dose-dependent fashion and anti-IL-6 antibody blocked this activity (Figure 4B). This result demonstrates that HOBIT CM contains bioactive IL-6. To determine the relevance of HOBIT IL-6 production to osteoblasts, we subjected POB CM to IL-6 ELISA. POB CM contained levels of IL-6 similar to those of HOBIT cells (Figure 4A).

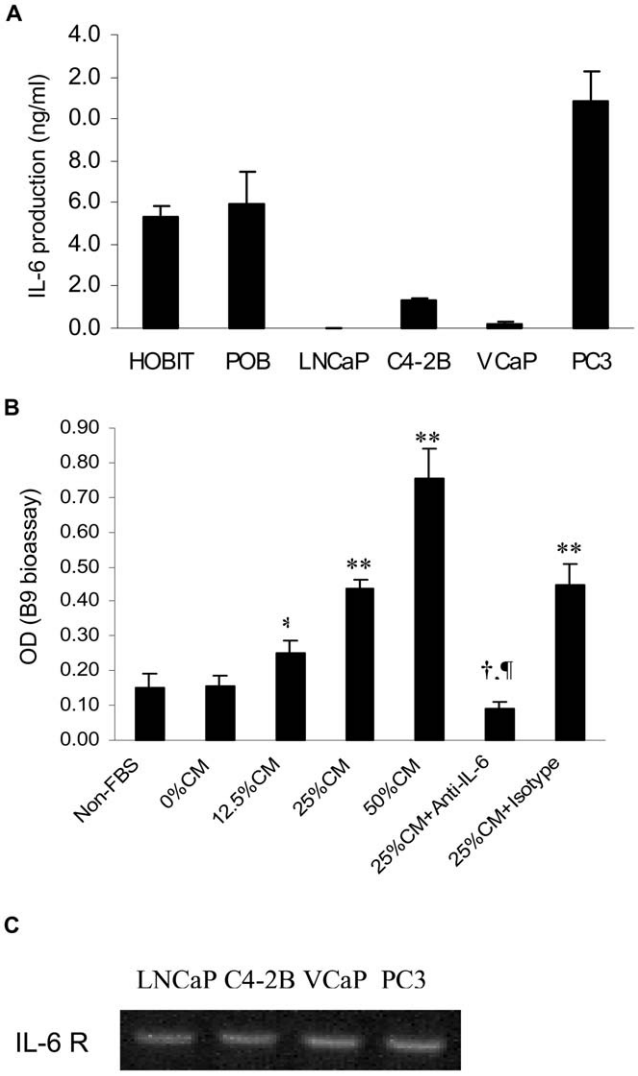

Figure 4. HOBIT CM and POB CM contain bioactive IL-6; LNCaP, C4-2B, and VCaP cells produce low levels of IL-6 and express IL-6R. (A) HOBIT CM and POB CM, and cell culture supernatant collected from LNCaP, C4-2B, VCaP, and PC3 were measured for IL-6 using a human ELISA. Data are represented as Mean ± SD from triplicate samples. (B) B9 bioassay was performed for measuring bioactive IL-6 in the HOBIT CM. Data are represented as Mean ± SD of the OD value from triplicate samples. $^{*}P < 0.01$ vs 0% CM-treated cells; $^{**}P < 0.001$ vs 0% CM-treated cells; $\dagger P < 0.001$ vs 25% CM-treated cells; $\P P < .01$ vs 0% CM-treated cells. (C) Total RNA was isolated from LNCaP, C4-2B, VCaP, and PC3 cell lysates. IL-6R mRNA expression was determined by RT-PCR. The PC3 cell line was used as a positive control for IL-6R expression. The PCR products were subjected to electrophoresis on a 1.5% agarose gel, stained with ethidium bromide.

CaP cells produce low levels of IL-6 and express IL-6 receptor

IL-6 has been shown to be produced at different levels by different CaP cell lines, and it functions through binding to its receptor on the CaP cells. It differentially regulates androgen receptor transactivation via PI3K-Akt, JNK/STAT3, and MAPK signal pathways [22]. To test if various CaP cell lines produce IL-6 we measured IL-6 expression in the supernatants. IL-6 levels were undetectable in LNCaP CM, whereas IL-6 levels were low compared to HOBIT and POB CM in the C4-2B and VCaP CM. PC3 CM had high levels of

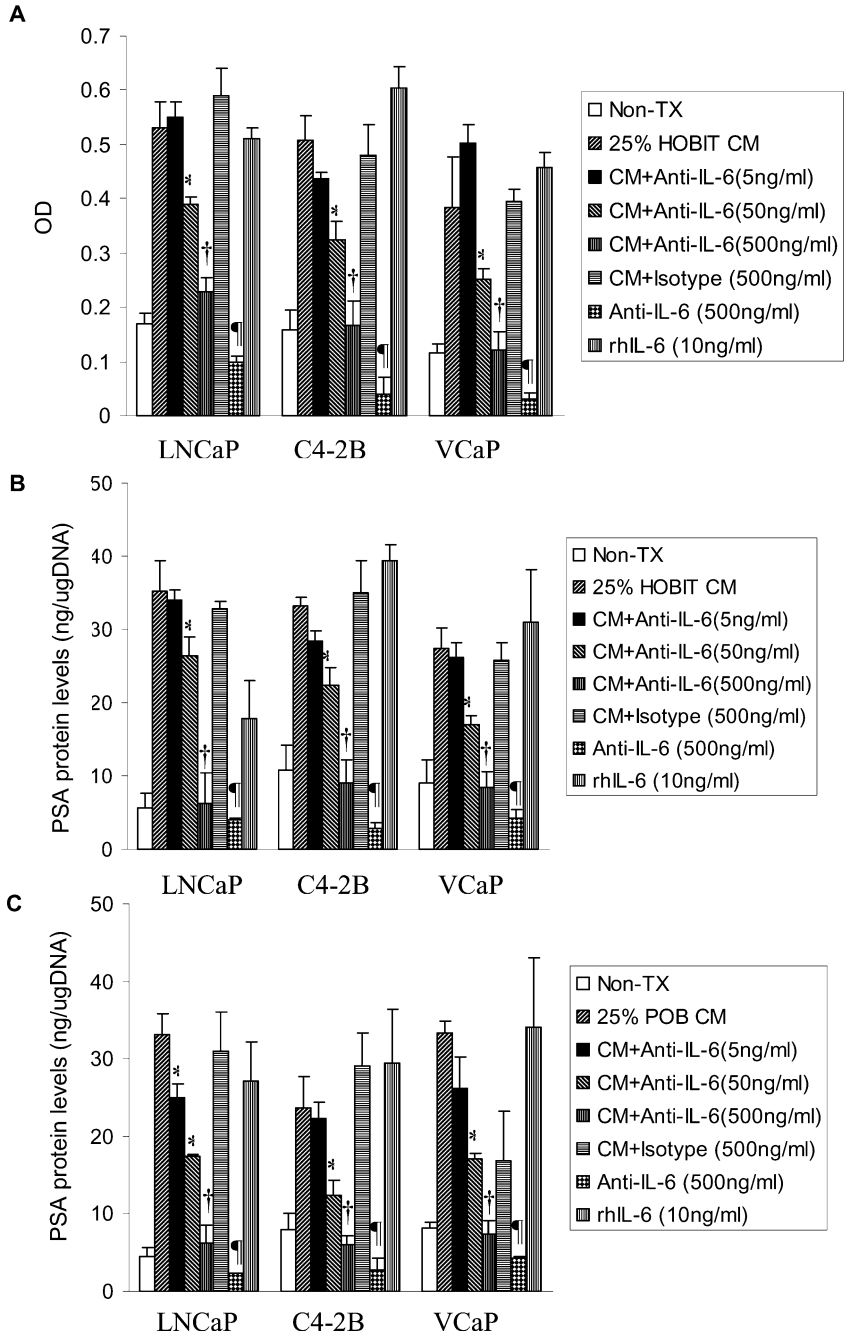

Figure 5. Anti-IL-6 antibody inhibits HOBIT CM- or POB CM-induced cell proliferation and PSA protein expression in LNCaP, C4-2B, and VCaP cells. (A) LNCaP, C4-2B and VCaP cells were seeded in triplicates at 5000 cells/well in their maintaining media in 96-well plate. Twelve hours later, the media was changed to DMEM supplemented with 0.5% charcoal-striped FBS. Then LNCaP, C4-2B, and VCaP cells were treated 25% HOBIT CM and/or indicated concentrations of anti-IL-6 antibody or isotype control (500 ng/ml), or recombinant human IL-6 (10 ng/ml). The cells were allowed to grow 48 hours, then the cell proliferation assay was performed using MTS assay. Data are presented as Mean ± SD of OD value. $^{*}P < 0.001$ vs non-treated cells; $\dagger P < 0.001$ vs 25%CM-treated cells; $\P P < 0.01$ vs non-treated cells. (B) LNCaP, C4-2B and VCaP cells in their maintaining media were added to the 12-well-plate at 3×10^5/well in triplicates. After 12 hours of culture, the media was changed to DMEM plus 0.5% CS-FBS and 25% HOBIT CM and/or indicated concentrations of anti-IL-6 antibody or isotype control (500 ng/ml), or recombinant human IL-6 (10 ng/ml). PSA protein in the culture supernatant was measured using the ACCUCYTE human PSA assay kit and normalized to total cellular DNA. Data are presented as Mean ± SD of OD value. $^{*}P < 0.001$ vs Non-treated cells; $\dagger P < 0.001$ vs 25%CM-treated cells; $\P P < 0.01$ vs non-treated cells. (C) LNCaP, C4-2B, and VCaP cells in their maintaining media were added to the 12-well-plate at 3×10^5/well in triplicates. After 12 hours of culture, the media was changed to DMEM plus 0.5% CS-FBS and 25% POB CM and/or indicated concentrations of anti-IL-6 antibody or isotype control (500 ng/ml), or recombinant human IL-6 (10 ng/ml). PSA protein in the culture supernatant was measured using the ACCUCYTE human PSA assay kit and normalized to total cellular DNA. Data are presented as Mean ± SD of OD value. $^{*}P < 0.001$ vs Non-treated cells; $\dagger P < 0.001$ vs 25%CM-treated cells; $\P P < 0.01$ vs non-treated cells.

IL-6 (Figure 4A). To determine if CaP cells can respond to IL-6, we determined if the IL-6R was present. We found that LNCaP, C4-2B, VCaP, and PC-3 cells express IL-6R mRNA as identified by RT-PCR (Figure 4C). Taken together, these results suggest that osteoblasts produce higher levels of IL-6 than CaP cells and that the CaP cells can respond to IL-6 through the IL-6R.

Anti-IL-6 antibody inhibits HOBIT-CM and POB-CM-induced CaP cells proliferation and PSA expression

To test whether IL-6 induces LNCaP, C4-2B and VCaP cell proliferation and PSA production *in vitro*, the cells were treated with recombinant IL-6. We found that IL-6 induced the proliferation of LNCaP, C4-2B, and VCaP cells (Figure 5A) and PSA production from these cells (Figure 5B). To test if HOBIT CM induced CaP proliferation and PSA production through IL-6, the CaP cells were grown in HOBIT CM supplemented with increasing doses of anti-IL-6 antibody. HOBIT CM induced LNCaP, C4-2B, and VCaP cell proliferation (Figure 5A) and PSA production (Figure 5B). Anti-IL-6 antibody inhibited the HOBIT CM-induced CaP cell proliferation (Figure 5A) and PSA production (Figure 5B) in a dose-dependent fashion. Additionally, anti-IL-6 alone reduced basal levels of LNCaP, C4-2B, and VCaP proliferation and PSA production (Figures 5A and B). To determine if these results extend to POB, we incubated the CaP cells with POB CM in the presence and absence of anti-IL-6 antibody. Similar to the HOBIT CM, POB CM induced LNCaP, C4-2B, and VCaP proliferation and PSA production and anti-IL-6 antibody blocked these activities (Figure 5C).

Anti-IL-6 antibody inhibits HOBIT-CM-induced AR nuclear translocation in C4-2B cells

We have previously demonstrated that recombinant IL-6 activates AR nuclear translocation in CaP cells [20]. To investigate whether HOBIT CM induces AR nuclear translocation through IL-6, various dose of neutralizing anti-IL-6 antibody was added to HOBIT CM (25%)-treated C4-2B cells that were stably transfected with an AR-GFP fusion vector. HOBIT CM induced AR translocation and neutralizing anti-IL-6 antibody dose-dependently inhibited HOBIT-CM-induced AR nuclear translocation in C4-2B cells (Figure 6A). These results indicate that osteoblast-derived IL-6 induces AR translocation in C4-2B cells.

Antiandrogen inhibits HOBIT-CM-induced CaP cell proliferation

Antiandrogens, such as BIC, were designed to treat advanced CaP through interfering with AR-mediated cell survival and through initiating cell death [30]. To test whether HOBIT CM-induced CaP cell proliferation through AR, we incubated LNCaP, C4-2B, and VCaP cells in HOBIT CM with the addition of BIC. We found that BIC inhibited HOBIT-CM-induced cell proliferation in a dose-dependent fashion in these cells (Figure 6B). These results demonstrate that HOBIT CM induces CaP proliferation through the AR.

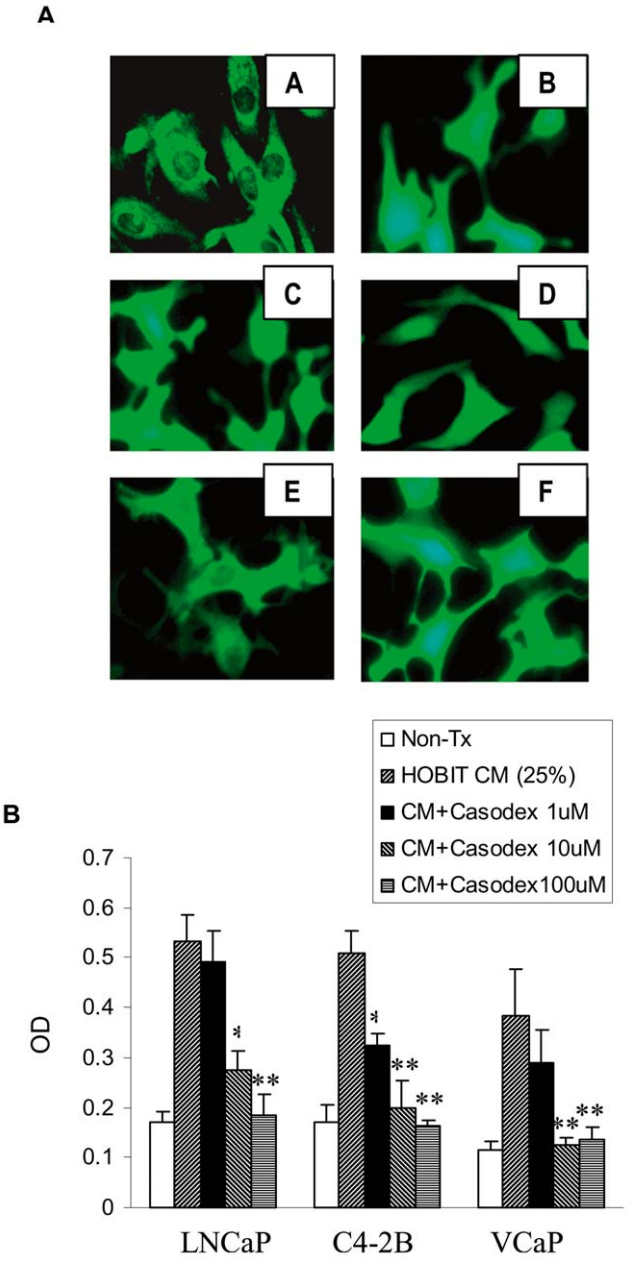

Figure 6. Osteoblast-derived IL-6 induces CaP cell proliferation through the androgen receptor (AR). (A) C4-2B cells in their maintaining media were added to the glass coverslips in 12-well-plate at 3×10^5/well. After 12 hours of culture, the media was changed to DMEM plus 0.5% CS-FBS and 25anti-IL-6 antibody or isotype control (500 ng/ml), or recombinant human IL-6 (10 ng/ml). Immunofluorescent imaging was captured after 48 hrs cell culture using a fluorescent microscope. Panel A: non-treated cells; panel B: 25% CM; panel C: 25% CM plus anti-IL-6 antibody (5 ng/ml); panel D. 25% CM plus anti-IL-6 antibody (50 ng/ml); panel E. 25% CM plus anti-IL-6 antibody (500 ng/ml); panel F: 25(500 ng/ml). (B) LNCaP, C4-2B and VCaP cells were seeded in triplicates at 5000 cells/well in their maintaining media in 96-well plate. Twelve hours later, the media was changed to DMEM supplemented with 0.5% charcoal-striped FBS. Then LNCaP, C4-2B, and VCaP cells were treated various concentrations of BIC. The cells were allowed to grow 48 hours, then the cell proliferation assay was performed using MTS assay. Data are presented as Mean ± SD of OD value from triplicates. $^{*}P < 0.01$ vs non-treated cells; $^{**}P < 0.001$ vs 25% CM-treated cells.

Discussion

CaP metastases selectively develop in bone [31–32]. It is unclear if the bone selectivity is due to the cancer cells' ability to home to bone or if the bone microenvironment is conducive to CaP cell growth. Most likely it is a combination of these activities. In the current study, we demonstrated that IL-6 is an osteoblast-derived factor that induces CaP cell proliferation through activation of the AR. This finding supports Paget's seed and soil hypothesis [3] through demonstrating that a factor produced in the bone microenvironment contributes to growth of cancer cells that selectively metastasize to bone.

In addition to its role as a key mediator of inflammation [15], IL-6 has pleiotropic effects in CaP cell biology [reviewed in 23]. It has been shown that IL-6 and its receptor are expressed on CaP cell lines [18–19] as we identified in two additional CaP cell lines, C4-2B and VCaP, in the current study. Furthermore, IL-6 serum levels are elevated in patients with hormone refractory CaP compared to those with localized disease [16–17]. However, the source of serum IL-6 is unknown. Although the CaP cells themselves produced IL-6, we found that the levels produced by osteoblasts were significantly higher, thus the major source of serum IL-6 is more likely from bone as opposed to the cancer cells. Furthermore, because the osteoblast-derived IL-6 is produced at a much higher level than that from the cancer cells themselves, it is possible that the osteoblast-derived IL-6 acts in paracrine manner in the CaP cells above and beyond the autocrine IL-6 activity. However, we cannot rule out that in the bone microenvironment CaP cells gain the ability to produce increased amounts of IL-6. It has been previously documented that CaP cells gain an osteoblast-like phenotype [33–34] which is consistent with the possibility that they also gain the ability to produce more IL-6. Measurement of serum human IL-6 in the mice implanted with tumor in the current study did not show any changes during tumor growth (data not shown). However, this may be due to the small volume of tumor compared to the whole animal and that IL-6 may have been diluted. At this point it is not clear if the bone microenvironment influences the ability of prostate cancer cells to produce IL-6.

The effect of IL-6 on CaP cell proliferation has generated contradictory results *in vitro*. For example, several studies demonstrated that IL-6 inhibits LNCaP cell proliferation [19, 21, 35] and induces the cells towards neuroendocrine differentiation [36–40]; whereas, other studies revealed that IL-6 induced cell growth [41]. The ability of IL-6 to promote differentiation of cancer cells may account for the decreased proliferation in some instances. In contrast, IL-6 induced androgen-independent CaP cell growth in several studies [18, 42], although, on one study, IL-6 had no effect on androgen-independent CaP cell proliferation [19]; however, in that same study, anti-IL-6 inhibited the proliferation of the androgen-independent lines suggesting that there was an autocrine proliferative effect that was maximally stimulated. Furthermore, IL-6 has been shown to have an anti-apoptotic effect on both basal and chemotherapy induced apoptosis of CaP cells [43–45]. In support of the *in vitro* data, the administration of anti-IL-6 antibody inhibited PC-3 CaP tumor growth *in vivo* [46]. The anti-IL-6 antibody administration was associated with increased apoptosis of tumor cells. In the current study, the observation that anti-IL-6 antibody blocked HOBIT-induced and POB-induced CaP proliferation and PSA production suggests that HOBIT and POB cells promote CaP proliferation and PSA production through IL-6. Additionally, the observation that anti-IL-6 antibody inhibited basal CaP cell proliferation and PSA expression, combined with our identification that these cells express both IL-6 and IL-6 receptor, suggests that these cells respond to IL-6 in an autocrine fashion. Taken together, our results indicate that the CaP cells respond to IL-6 in both an autocrine and paracrine fashion, which suggests that osteoblast-derived production of IL-6 in the bone microenvironment favors CaP growth or survival. These data demonstrate that IL-6 has complex effects on CaP and suggest IL-6 contributes to the progression of both androgen-dependent and androgen-independent cancer *in vitro*.

The mechanisms through which IL-6 modulates CaP cell activity are not clear. IL-6 has been reported to activate multiple signaling pathways in CaP cells including the PI-3 kinase [45], Jak/Stat [47], and MAP kinase [48] pathways. Initiation of these pathways results in activation of the AR in some instances. IL-6 has been demonstrated to activate the AR in the absence of androgen resulting in PSA production [20–22]. Consistent with these previous reports, we identified, in the current study, that HOBIT CM-induced AR nuclear translocation through IL-6. Proof of the functional importance of the AR induction was provided by the demonstration that the anti-androgen BIC, which completely blocks AR function [49], blocked HOBIT CM-induced CaP cell proliferation. This is consistent with previous reports in which BIC has been shown to block IL-6-induced AR nuclear translocation [20] and PSA production [20–21]. These data suggest that osteoblasts can provide the signal necessary to activate the AR and promoter CaP progression in bone.

The bone microenvironment is rich in many growth factors that have the potential to promote cancer growth in bone [reviewed in 5]. It has been postulated that tumor-induced osteolysis results in release of growth factors, such as transforming growth factor-β, that are sequestered in the bone matrix [reviewed in 50]. The current study demonstrates that, in addition to these matrix-derived factors, cells in the bone microenvironment can produce factors that promote cancer cell growth in bone.

In conclusion, the current study demonstrated that IL-6 is a key mediator of osteoblast-induced CaP cell proliferation and PSA expression through activation of the AR. This finding supports the idea that cross-talk between the bone microenvironment and CaP cells promotes tumor establishment and progression in bone. Identifying the factors that mediate cross-talk between the bone microenvironment and tumor may lead to identification of therapeutic targets. Accordingly, this study suggests that further exploration of

IL-6 as a putative target to diminish the development and progression of bone metastases is warranted.

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