Bone Morphogenetic Protein Signaling in Prostate Cancer Cell Lines

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Abstract   Prostate cancer is the most commonly diagnosed malignancy in men and is often associated with bone metastases. Prostate cancer bone lesions can be lytic or schlerotic, with the latter predominating. Bone morphogenetic proteins (BMPs) are a family of growth factors, which may play a role in the formation of prostate cancer osteoblastic bone metastases. This study evaluated the effects of BMPs on prostate cancer cell lines. We observed growth inhibitory effects of BMP-2 and -4 on LNCaP, while PC-3 was unaffected. Flow cytometric analysis determined that LNCaP cell growth was arrested in G_1 after bone morphogenetic protein-2 treatment. Treatment of LNCaP and PC-3 with BMP-2 and -4 activated downstream signaling pathways involving SMAD-1, up-regulation of $p21^{CIP1/WAF1}$ and changes in retinoblastoma (Rb) phosphorylation. Interestingly, bone morphogenetic protein-2 treatment stimulated a 2.7-fold increase in osteoprotegerin (OPG), a molecule, which inhibits osteoclastogenesis, production in PC-3. J. Cell. Biochem. 91: 151–160, 2004.
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Bone and lymph nodes are the two most common sites for prostate cancer (CaP) metastasis during advanced stages of disease. CaP bone metastases are often associated with new bone formation and increased bone density. A family of growth factors, which may play a role in CaP stimulation of bone synthesis is the bone morphogenetic proteins (BMPs). BMPs are secreted signaling molecules belonging to the transforming growth factor-β (TGF-β) superfamily [Hogan, 1996]. They were originally characterized as inducers of bone formation at extraskeletal sites in vivo [Reddi, 1998]. Numerous studies have demonstrated additional roles for BMPs in embryogenesis and organogenesis [Lyons et al., 1989; Jones et al., 1991], as well as regulators of cell growth, differentiation, migration, and apoptosis [Graham et al., 1994; Lind et al., 1996; Li et al., 1998].

Members of the TGF-β superfamily, such as the BMPs, initiate-signaling pathways through binding two types of serine/threonine transmembrane receptor kinases, type I and type II. There are three type I receptors, which bind the various members of the BMP family. They are BMPR IA (ALK-3), BMPR IB (ALK-6), and type I activin receptor (ALK-2) [Koenig et al., 1994; ten Dijke et al., 1994]. The type II receptors are BMPR II and the activin receptors IIA and IIB [Liu et al., 1995; Yamashita et al., 1995]. BMPs bind the type II receptor which in turn dimerizes with and phosphorylates the type I receptor. The type I receptor then phosphorylates and activates members of the SMAD family of proteins, particularly SMAD-1, -5, and -8 [Kretzschmar et al., 1997; Zhao and Hogan, 1997; Nishimura et al., 1998]. These SMADs interact with SMAD-4 resulting in nuclear translocation, where they interact with transcription factors and target various genes.

Message for BMP-2, -3, -4, and -6 have been reported in normal and cancerous prostate tissues [Harris et al., 1994; Autzen et al., 1998], and BMP-4 and -7 have been demonstrated to

play a role in prostate development [Thomas et al., 1998; Lamm et al., 2001]. In addition, BMP-4, -6, and -7 were detected in prostate skeletal metastases [Autzen et al., 1998; Thomas et al., 1998, 2000; Masuda et al., 2003] suggesting that prostate-expressed BMPs play a role in the formation of osteoblastic lesions. Furthermore, BMP receptors were detected in prostate tissues and cell lines [Ide et al., 1997a; Kim et al., 2000; Brubaker et al., 2001]. Therefore, BMPs expressed by both tumor and bone cells may exhibit direct effects on CaP cells. Ide et al. [1997b] demonstrated that BMP-2 inhibited growth of LNCaP, an androgen-sensitive prostate cell line, while the androgen-independent lines PC-3 and DU 145 were unaffected. BMP-2 was also reported to inhibit growth of breast cancer cell lines [Ghosh-Choudhury et al., 2000a,b; Pouliot and Labrie, 2002], BMP-2 and -4 were found to stimulate apoptosis of human myeloma cells [Kawamura et al., 2000, 2002; Hjertner et al., 2001], and Franzen and Heldin [2001] reported that BMP-7 inhibited growth of anaplastic thyroid carcinoma.

This study was undertaken to evaluate the downstream signaling pathways activated by BMP-2 and -4 in CaP cell lines LNCaP and PC-3. We observed inhibition of LNCaP growth via pathways involving the cyclin dependent kinase inhibitor p21$^{CIP1/WAF1}$. Interestingly, we also detected a 2.7-fold increase in osteoprotegerin (OPG), a factor which inhibits osteoclastogenesis, after BMP-2 treatment of PC-3 cells.

MATERIALS AND METHODS

Cell Culture and Supplements

Tissue culture media and supplements were obtained from Gibco Life Technologies, Inc. (Rockville, MD). CaP cell lines were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) under standard culture conditions. The CaP cell lines used in this study were DU 145, PC-3, LNCaP (ATCC, Manassas, VA) and LNCaP sublines, C4 and C4-2B (Urocore, Oklahoma City, OK). Recombinant human BMP-2 and -4 were generously provided by the Genetics Institute (Cambridge, MA) a subsidiary of Wyeth (Madison, NJ).

BMPR Western Blotting

CaP cell lines (DU 145, PC-3, LNCaP, C4, C4-2B) cultured for 5 days on 100 mm dishes were lysed in 500 μl lysis buffer (10 mM Tris, pH 7.4,

50 mM NaCl, 0.25% Triton X-100, 0.25% sodium deoxycholic acid, 0.5% CHAPS, Complete™ protease inhibitors) and the protein content was measured using the Bio-Rad DC Protein assay (Bio-Rad Laboratories, Hercules, CA). Proteins (30 μg/well) were separated on 10% SDS–PAGE and transferred to PVDF membranes. Blots were blocked in a 1:1 solution of NaP-Sure blocker (Geno Technology, Inc., St. Louis, MO) and Tris-buffered saline + 0.1% Tween-20 (TBS-T) for 2 h, then probed with affinity purified goat polyclonal antibodies raised against the extracellular domains of BMPR IA, BMPR IB, and BMPR II (R&D Systems, Minneapolis, MN) at 0.2 μg/ml for 2 h at room temperature. After thorough rinses, immunoreactivity was detected using a donkey anti-goat antibody conjugated to HRP (1:50,000; Chemicon, Temecula, CA) and ECL (Amersham, Piscataway, NJ) reagents.

Proliferation Studies

LNCaP (4,000 cells/well) and PC-3 (2,000 cells/well) were seeded in 96-well plates in 100 μl 2% FBS, phenol red free RPMI 1640. The following day, cells were treated with 0, 10, 50, 100, 250, and 500 ng/ml BMP-2, or 0, 1, 3, 10, 30, and 100 ng/ml BMP-4 for 3 days. For competition studies, 3 μg/ml soluble BMPR IB (R&D Systems) was added to wells in the presence of 10 ng/ml BMP-4. Cell proliferation was evaluated using the Quick Cell Proliferation Assay (BioVision, Inc., Mountain View, CA). Statistical significance ($P < 0.05$) was evaluated using a one-way ANOVA and a Dunnett's multiple comparison post-test [Dunnett and Tamhane, 1991].

Cell-Cycle Studies

LNCaP (500,000 cells/well) and PC-3 (250,000 cells/well) were seeded in 6-well plates in 10% FBS, RPMI 1640. The following day, the media were changed to 2% FBS in phenol red free RPMI 1640 supplemented with 0 or 250 ng/ml BMP-2. After 1, 2, and 3 days in culture, cells were trypsinized and stained with 10 μg/ml DAPI (Sigma, St. Louis, MO) in the presence of 1% Nonidet-P-40 for flow cytometric analysis [Douglas et al., 1998]. Flow cytometry was done with an Influx flow cytometer (Cytopeia, Seattle, WA) on 20,000 nuclei per sample. Cell-cycle analysis was performed using Wincycle software (Phoenix Flow System, San Diego, CA).

Statistical significance ($P < 0.05$) was evaluated using a Student's t-test.

SMAD-1 Signaling

LNCaP (10×10^6) and PC-3 (5×10^6) were cultured in T162 culture plates for 4–6 days in 10% FBS RPMI 1640. The media were changed to 2% FBS in phenol red free RPMI 1640, and cells were treated with BMP-2 (250 ng/ml) or BMP-4 (10 ng/ml) for various time points over a 24 h time course. Cells were scraped in Hank's balanced salts solution for isolation of nuclear and cytosolic fractions as previously reported [Brubaker et al., 2003]. Thirty-five micrograms of nuclear proteins were separated on 10% SDS–PAGE and transferred to PVDF membranes. The blots were blocked with NapSure blocker (2 h), and total and phosphorylated SMAD-1 levels were detected using rabbit polyclonal antibodies from Upstate Biotechnology, Inc. (UBI, Waltham, MA, catalog numbers 06-653 (0.75 µg/ml) and 06-702 (0.5 µg/ml), respectively). A donkey anti-rabbit antibody conjugated to HRP (Amersham, 1:2,000) was used for detection in concert with the Amersham ECL detection kit. Densitometry values were determined using the Alpha Imager 2200 Documentation and Analysis System (Alpha Innotech Corp., San Leandro, CA).

p21$^{CIP1/WAF1}$, p27^{KIP1}, and Retinoblastoma (Rb) Western Blots

LNCaP (500,000 cells/well) and PC-3 (250,000 cells/well) were seeded in 6-well plates in 10% FBS in RPMI 1640. The following day, the media were changed to 2% FBS in phenol red free RPMI 1640 supplemented with 0, 125, or 250 ng/ml BMP-2. Cells were lysed in 250 µl lysis buffer (10 mM Tris, pH 7.4, 50 mM NaCl, 0.25% Triton X-100, 0.25% sodium deoxycholic acid, 0.5% CHAPS, Complete protease inhibitors) at 1, 2, and 3 days. Forty micrograms of protein were separated on 15% (for p21 and p27) or 7.5% (for Rb) SDS–PAGE and transferred to PVDF membranes. After blocking with NapSure blocker, membranes were probed with either mouse anti-p21$^{CIP1/WAF1}$ (1 µg/ml, UBI) or rabbit anti-p27^{KIP1} (1 µg/ml, UBI) or a mixture of three polyclonal antibodies against phosphorylated-Rb (1:1,000 each, anti-phospho-Rb (Ser 780, Ser 795, Ser 807/811), Cell Signaling Tech., Inc., Beverly, MA) for 1 h. Signal was detected using either donkey anti-mouse or anti-rabbit antibodies conjugated to HRP (1:2,000, Amersham) in conjunction with Amersham ECL reagents. To confirm uniform loading of protein, blots were re-probed with an anti-actin rabbit polyclonal antibody (1:1,000, Sigma). Densitometry values were determined using the Alpha Imager 2200 Documentation and Analysis System (Alpha Innotech Corp.).

OPG ELISA

LNCaP (500,000 cells/well) and PC-3 (250,000 cells/well) were seeded in 6-well plates in 10% FBS, RPMI 1640. The following day, the media were changed to 2% FBS in phenol red free RPMI 1640 supplemented with 0 or 250 ng/ml BMP-2. Culture media were collected after 3 days for the determination of OPG levels using an ELISA kit (Biomedica, Southbridge, MA). The cells were trypsinized and counted for normalization of OPG levels to cell number (pg OPG/10^6 cells/ml). Statistical significance ($P < 0.05$) was determined using the Student's t-test.

RESULTS

BMPR Expression and Growth Inhibition

Using antibodies against the BMPRs, we detected BMP type IB receptor in LNCaP and its sublines, C4 and C4-2B, while DU 145 and PC-3 were negative for its expression (Fig. 1A). BMPR IA was not detected in any of the CaP cell lines tested, even though it was present in CaP tissues (data not shown). BMPR II protein was detected in all CaP lines tested (Fig. 1B). Growth of the androgen-sensitive CaP cell line, LNCaP, was significantly inhibited ($P < 0.05$) by BMP-2 and -4, while PC-3, an androgen-independent CaP cell line, was not affected (Fig. 2). Cell-cycle analysis using DAPI staining demonstrated that BMP-2 treatment resulted in accumulation of LNCaP in the G_1 phase of the cell cycle. After 2 days of BMP-2 treatment, 88.7% of the LNCaP cells were arrested in G_1, as compared to 69.0% of the cells in G_1 for the untreated LNCaP control (Fig. 2). BMP-2 treatment did not affect the cell-cycle distribution of PC-3 cells.

BMP Signaling and Downstream Effects

SMAD-1 is a signaling molecule that is phosphorylated upon BMP ligand binding to its receptor complex. SMAD-1 phosphorylation was detected in LNCaP and PC-3 after
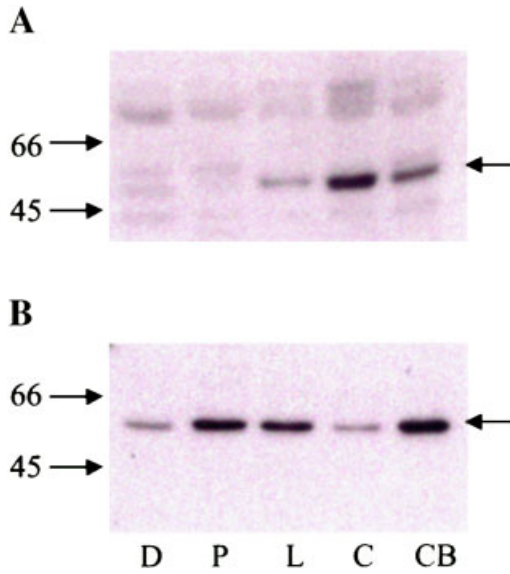
A

B

Fig. 1. Expression of BMPR IB and BMPR II protein in CaP cell lines. **A**: BMPR IB was detected in LNCaP (L) and its sublines, C4 (C), and C4-2B (CB), as a 48 kDa band (arrow), but was absent in DU 145 (D) or PC-3 (P) lysates. **B**: The antibody against BMPR II labeled a 50 kDa band (arrow) in all the CaP cells tested. BMPR IA was not detected in CaP cell lines (blot not shown). Molecular weights markers are labeled to the **left side** of the blots and are in kiloDaltons.

treatment with BMP-2 and -4 (Fig. 3). SMAD-1 activation demonstrates intact BMP receptor signaling in both the LNCaP and PC-3 cell lines. SMAD-1 activation stimulates transcription of various effector genes including those involved in cell growth and differentiation.

Cyclin-dependent kinase inhibitors (CKIs) such as $p21^{CIP1/WAF1}$ and $p27^{KIP1}$ are effector proteins involved in growth arrest induced by many growth factors including TGF-β and BMPs [Li et al., 1995; Jernvall et al., 1998]. CKIs bind cyclin-dependent kinases (CDKs) to inhibit their activity, thus inducing G_1-phase arrest. We observed up-regulation of $p21^{CIP1/WAF1}$ levels in both LNCaP and PC-3 after treatment with BMP-2 for 24, 48, and 72 h (Fig. 4). The levels of $p27^{KIP1}$ showed a slight increase in both cell lines at day three of treatment, but the increase was not significant. Downstream of the CDKs is the Rb protein, which regulates the transition from G_1 to S phase of the cell cycle. Rb is a substrate for the CDKs. When Rb is hyper-phosphorylated the cells can enter S phase and proliferate, while hypo-phosphorylated Rb causes arrest in G_1 [Buchkovich et al., 1989; Mihara et al., 1989]. We observed a shift towards the hypo-phos-
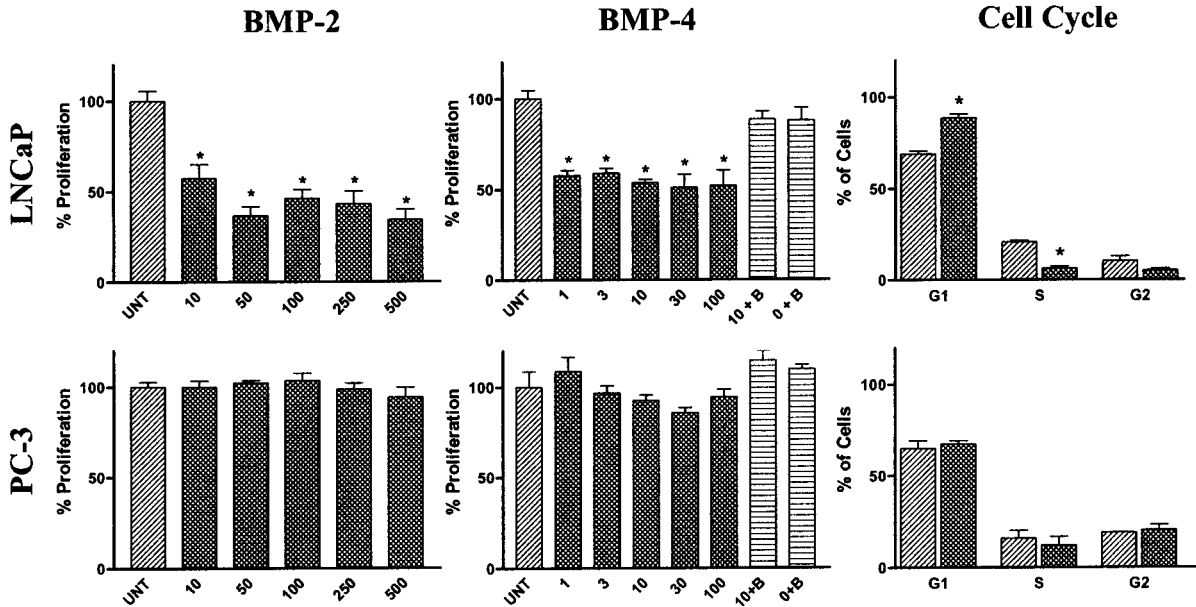

Fig. 2. The effects of bone morphogenetic protein (BMP)-2 and -4 on LNCaP and PC-3 proliferation and cell cycle. LNCaP treated with either BMP-2 or -4 for 3 days were growth inhibited, while PC-3 cells were unaffected. Treatment units are ng/ml. The (*) denotes treatments which were significantly ($P < 0.05$) different from the untreated control. Co-incubation with 3-μg/ml soluble BMPR IB (B) blocked the growth inhibition of LNCaP.

BMP-2 (250 ng/ml) caused growth arrest of LNCaP in the G_1 phase of cell cycle after 2 days of treatment. The fraction of LNCaP treated with BMP-2 in G_1 and S phases were significantly ($P < 0.05$) different from the untreated control cells (*). BMP-2 treatment did not affect the cell-cycle profile of PC-3. The untreated cells are marked by (▨) and BMP-2 treated cells are the (▰) boxes.
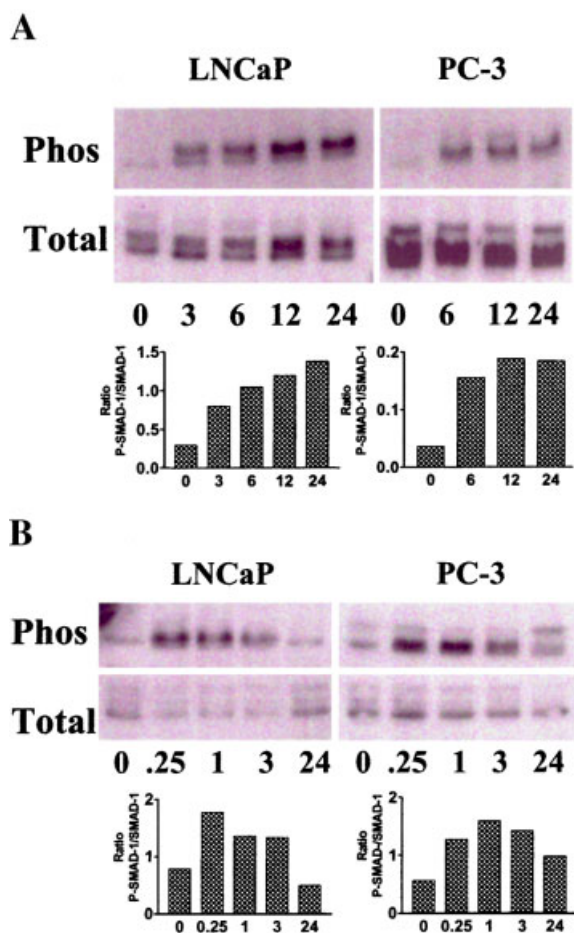
A

Fig. 3. SMAD-1 signaling stimulated by BMP-2 and -4. SMAD-1 phosphorylation (Phos) was detected in LNCaP and PC-3 cell lines treated with BMP -2 (**A**, 200 ng/ml) and -4 (**B**, 10 ng/ml) for various times (0.25–24 h) of treatment. Total SMAD-1 (total) levels demonstrated that the increased P-SMAD-1 levels were not due to increased total SMAD-1 expression. The ratios of P-SMAD-1/total SMAD-1 densitometry levels are shown below each blot.

phorylated state of Rb in LNCaP after treatment with BMP-2 for 24–72 h (Fig. 5), while no changes in Rb phosphorylation levels were detected in PC-3.

BMP Up-Regulation of OPG

BMPs stimulate bone formation through up-regulation of various bone related factors, such as collagen type I, osteopontin, osteocalcin, and OPG in osteoblasts [Hofbauer et al., 1998; Huang et al., 2002] via transcriptional regulation by SMADs and Runx2 [Lee et al., 2000; Ebara and Nakayama, 2002]. BMP-2 treatment up-regulated OPG levels in the media of PC-3 2.7-fold, while levels were decreased 1.8-fold in LNCaP (Fig. 6).

DISCUSSION

Unraveling the mechanisms involved in CaP stimulation of bone formation is imperative since CaP bone metastases are responsible for most of the morbidity and mortality associated with advanced stages of CaP. Expression of BMPs in CaP has been established with increased levels of BMP-4, -6, and -7 reported in CaP bone metastases. Since CaP expresses the receptors for BMPs, it is conceivable that BMPs present in the bone environment play a role in regulating CaP cell growth and/or expression of critical factors involved in bone remodeling.

BMPs play a role in development and the control of proliferation and differentiation. It has been reported that BMPs inhibit proliferation of cells from various tissues including aortic smooth muscle [Wong et al., 2003], developing prostate epithelial cells [Lamm et al., 2001], embryonic lung [Bragg et al., 2001], breast cancer cell lines [Pouliot and Labrie, 2002], anaplastic thyroid carcinoma cells [Franzen and Heldin, 2001], and B cells [Ishisaki et al., 1999]. Therefore, the expression of BMPs and its receptors in prostate epithelial cells may be involved in the control of proliferation of these cells. However, tumor cells typically escape from growth control. Soda et al. [1998] reported that BMP-2 inhibited growth of 16 out of 65 primary specimens of breast, ovarian, non-small cell lung, and prostate cancers, implying that the growth inhibitory pathways associated with BMP functions may be altered in cancer cells. We demonstrate that the androgen-sensitive, differentiated LNCaP cell line was growth inhibited by BMP-2 and -4 through G_1 cell-cycle arrest. In contrast, proliferation of the more aggressive de-differentiated PC-3 cell line was unaffected, and the cell-cycle distribution was unchanged. Since PC-3 was isolated from a CaP bone metastasis, signaling pathways associated with growth inhibition may have changed to allow survival of these cells in an elevated BMP environment. We have shown that LNCaP express BMPR IB, while this receptor was not detected in PC-3. We hypothesized that the resistance of PC-3 to BMP-2 may be due to the absence or low levels of BMPR IB. To investigate this hypothesis, we examined signaling pathways downstream of the BMPRs. In contrast to the differences in growth regulation by BMPs on LNCaP and PC-3, we found that SMAD-1 was activated in LNCaP and PC-3
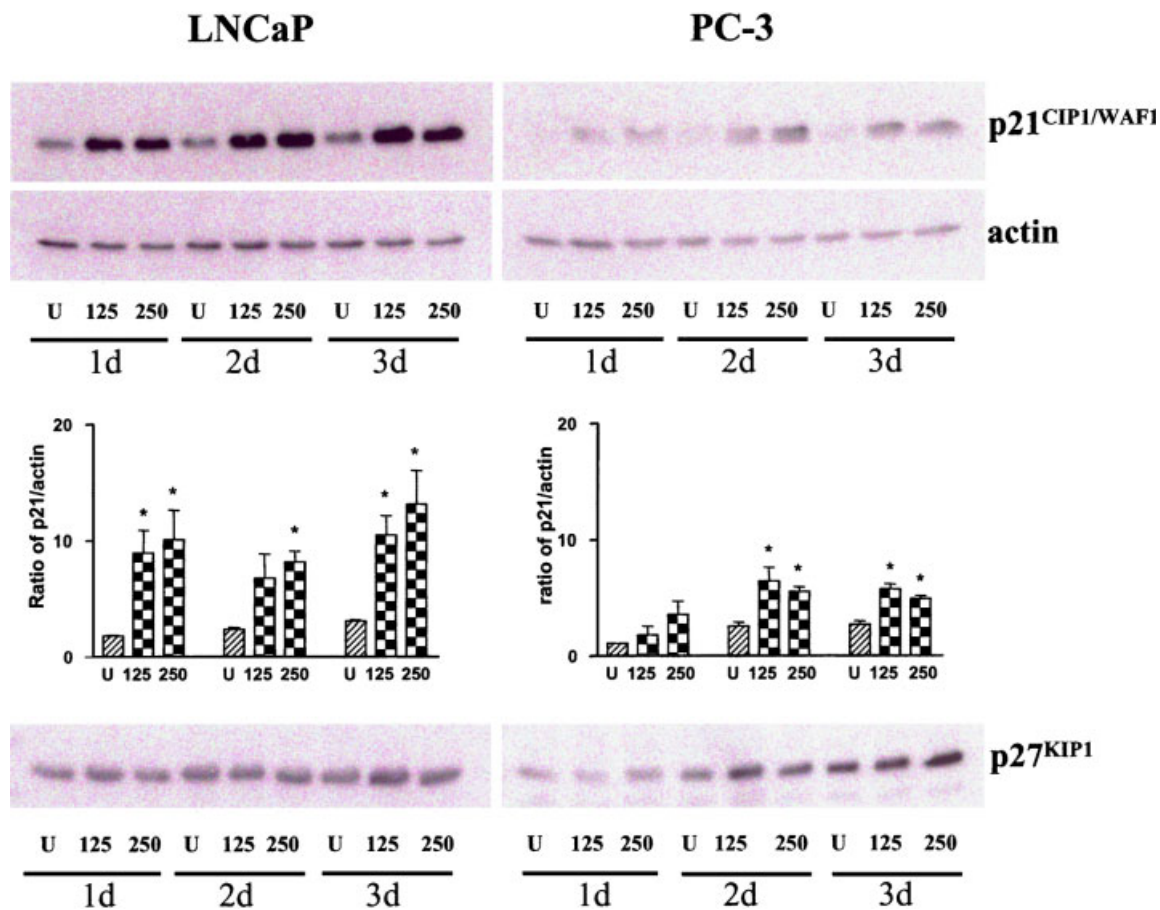
Fig. 4. BMP-2 treatment up-regulated p21[CIP1/WAF1] in LNCaP and PC-3. BMP-2 treatment (125 or 250 ng/ml) for 24, 48, and 72 h up-regulated p21[CIP1/WAF1] protein levels in LNCaP and PC-3 as compared to untreated (U) cells. p27[KIP1] levels were slightly increased by BMP-2 at 3 days of treatment, but the levels were not significant. Blots were probed for actin as a control for loading. Ratios of p21[CIP1/WAF1] to actin levels are presented in the graphs below the blots. The (*) denotes samples which were significantly ($P < 0.05$) different from the untreated. The data are the average of densitometry values taken from two separate experiments.
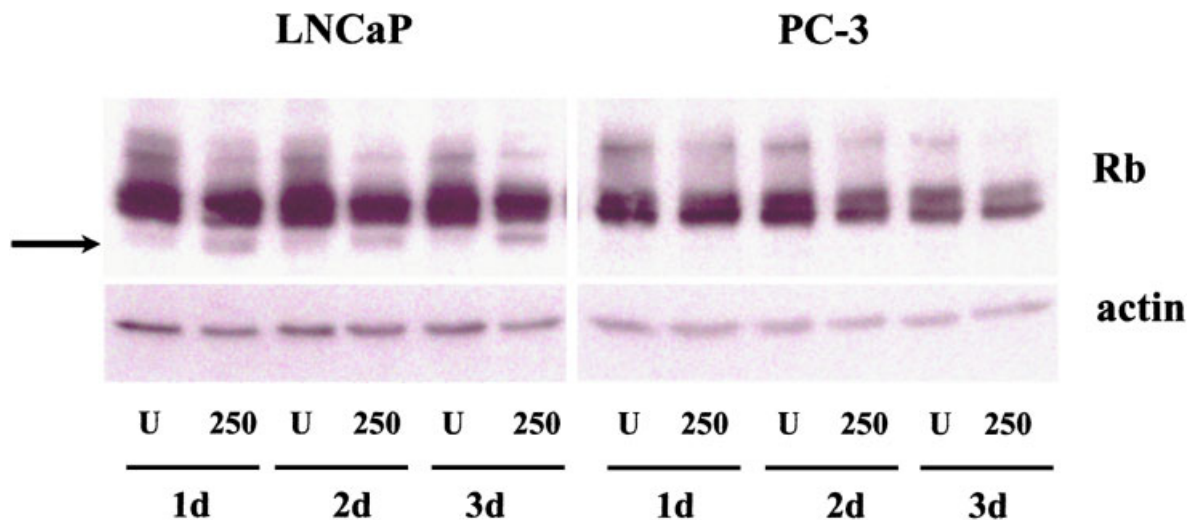

Fig. 5. BMP-2 effects on Rb phosphorylation levels in LNCap and PC-3. BMP-2 treatment for 24, 48, and 72 h prompted a shift in the levels of phosphorylated Rb towards the hypophosphorylated (arrow) state in LNCaP. This shift was not observed in the PC-3 cells. Actin was run as a control for loading.
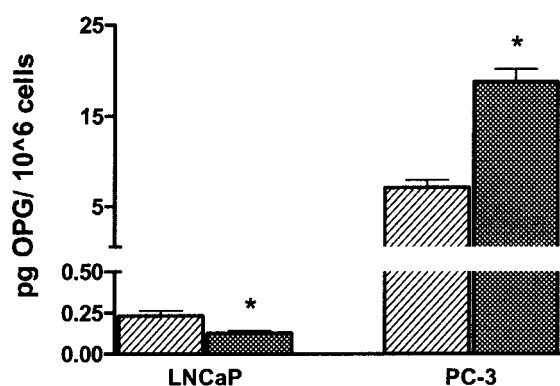
Fig. 6. BMP-2 treatment stimulated osteoprotegerin (OPG) production in PC-3. OPG levels were measured in the media of LNCaP and PC-3 cells treated with or without BMP-2 (250 ng/ml) for 3 days. BMP-2 stimulated a 2.7-fold increase in OPG production in PC-3, while a 1.8-fold decrease in OPG levels was observed with LNCaP. OPG levels were significantly up-regulated ($P < 0.05$) in PC-3 over untreated cells (*), while significantly down-regulated in LNCaP ($P < 0.05$). Untreated samples are represented by (▨), while BMP-2 treated cells are marked by (▦).

corroborating intact BMP signaling in both of these cell lines.

BMP-associated growth inhibitory responses are transmitted, at least in part, by the CDK inhibitor, p21$^{CIP1/WAF1}$. We found that BMP-2 treatment increased levels of p21$^{CIP1/WAF1}$ in LNCaP and caused further downstream inactivation of the Rb protein. Interestingly, p21$^{CIP1/WAF1}$ levels increased in PC-3 after BMP-2 treatment even though proliferation was unaffected. Also, the levels of phosphorylated Rb in PC-3 were unchanged by BMP-2 treatment, implying a change in the signaling downstream of the CKIs.

BMPs regulate the expression of various genes associated with osteoblast differentiation, in addition to its growth regulatory effects. Even though BMP-2 and -4 did not affect PC-3 proliferation, BMP-2 treatment stimulated expression of OPG in PC-3, while OPG levels decreased in LNCaP. OPG is a decoy receptor for the receptor activator for NF-κB ligand (RANKL), a molecule which stimulates osteoclastogenesis and lymphocyte development [Anderson et al., 1997; Simonet et al., 1997; Lacey et al., 1998]. Zhang et al. [2001] reported that OPG inhibited CaP-stimulated osteoclast formation. Production of OPG in CaP bone metastases in response to BMPs provides us with a model for the increased bone formation associated with CaP bone metas-

tases, through inhibition of osteoclastogenesis. In line with these findings, we have published several studies indicating increased levels of OPG in serum [Brown et al., 2001a] and bone metastases [Brown et al., 2001b] of advanced CaP patients. However, the mechanisms involved in the formation of CaP osteoblastic lesions cannot be as simple as up-regulation of OPG, since PC-3 produces highly osteolytic lesions [Corey et al., 2002]. The remodeling pathways involving OPG in PC-3 may well be overridden by factors which stimulate bone resorption such as matrix metalloproteinases [Nemeth et al., 2002], urokinase plasminogen activator [Yoshida et al., 1994], RANKL [Brown et al., 2001b], and PTHrP [Iwamura et al., 1994] expressed by PC-3.

Transcriptional regulation of bone-related genes by BMPs is mediated via the SMAD transducers and Runx2 [Hanai et al., 1999; Lee et al., 2000; Bae et al., 2001]. We and others have shown that PC-3 express high levels of Runx2 [Yeung et al., 2002; Brubaker et al., 2003], a transcription factor which regulates expression of bone related factors in osteoblasts [Ducy et al., 1997; Sato et al., 1998; Javed et al., 2001; Kern et al., 2001], including OPG [Thirunavukkarasu et al., 2000], while Runx2 transcriptional activity was not detected in LNCaP cells. We hypothesize that BMP-2 up-regulation of OPG in PC-3 is mediated through Runx2 and SMAD transcriptional regulation.

In summary, we have demonstrated that BMP-2 and -4 elicit diverse growth effects on CaP cells depending on their differentiation status. The growth regulatory effects of BMPs may be overcome by advanced CaP through alterations in the associated signaling pathways, as we observed for the differences in BMPR expression, as well as Rb phosphorylation levels in LNCaP and PC-3. Since CaP bone metastases express Runx2 [Brubaker et al., 2003], which interacts with SMADs, BMP signaling appears to play a role in increased levels of OPG, which favors decreased osteolysis and, therefore, development of osteoblastic lesions.

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