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IGF-I and MAP Kinase Involvement in the Stimulatory Effects of LNCaP Prostate Cancer Cell Conditioned Media on Cell Proliferation and Protein Synthesis in MC3T3-E1 Osteoblastic Cells

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Abstract Bone metastases from prostate cancer cause abnormal new bone formation, however, the factors involved and the pathways leading to the response are incompletely defined. We investigated the mechanisms of osteoblast stimulatory effects of LNCaP prostate carcinoma cell conditioned media (CM). MC3T3-E1 osteoblastic cells were cultured with CM from confluent LNCaP cells. LNCaP CM stimulated MAP kinase, cell proliferation (^{3}H-thymidine incorporation), and protein synthesis (^{14}C-proline incorporation) in the MC3T3-E1 cells. The increases in cell proliferation and protein synthesis were prevented by inhibition of the MAP kinase pathway. IGF-I mimicked the effects of the CM on the MC3T3-E1 cells and inhibition of IGF-I action decreased the LNCaP CM stimulation of ^{3}H-thymidine and ^{14}C-proline incorporation and MAP kinase activity. The findings indicate that IGF-I is an important factor for the stimulatory effects of LNCaP cell CM on cell proliferation and protein synthesis in osteoblastic cells, and that MAP kinase is a component of the signaling pathway for these effects. J. Cell. Biochem. 90: 925–937, 2003. © 2003 Wiley-Liss, Inc.

Key words: osteoblastic metastases; prostate cancer; IGF-I; MAP kinase; LNCaP cells

Skeletal metastases are a frequent consequence of prostate and breast cancer. In metastatic prostate cancer, the primary site for metastasis is the skeleton, with 70–80% of prostate cancer patients developing skeletal metastases [Chirardo, 1991]. Prostate cancer spreads most commonly to the well-vascularized areas of the skeleton such as the vertebral column, ribs, skull, and the proximal ends of the long bones [Carlin and Andriole, 2000]. Skeletal metastases can have painful and debilitating consequences, resulting in decreased quality of life for patients. The structural changes in bone resulting from skeletal metastases can cause severe bone pain, bone marrow suppression, leukopenia, hypercalcemia, and pathologic fractures [Chirardo, 1991].

Clinically, most skeletal metastases result in mixed osteoblastic and osteolytic lesions. However, prostate cancer primarily elicits osteoblastic metastases and abnormal bone formation. Bone metastases from prostate cancer have been described as osteosclerotic, with increased trabecular bone volume and newly woven bone [Charhon et al., 1983], a less organized bone that develops in response to injury and is associated with accelerated osteoblastic activity [Turner, 1992]. The woven bone generated by the osteoblastic metastasis is composed of loosely packed and randomly oriented collagen bundles that results in bone with decreased strength and increased susceptibility to fracture [Rosol, 2000; Keller et al., 2001]. Recent studies have shown that osteoblastic metastases form at sites of previous osteoclastic resorption, indicating that bone resorption might be required to initiate subsequent osteoblastic bone formation [Carlin and Andriole, 2000; Keller et al., 2001; Zhang et al., 2001].

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The cellular and molecular mechanisms underlying the formation of osteoblastic metastases are not completely understood. The abnormal bone growth elicited by prostate cancer could be attributable either to factors produced by the tumor cells, to factors released by the bone matrix or to interactions between these factors [Charhon et al., 1983; Guise and Mundy, 1998]. Soluble factors produced by the prostate cancer cells can promote proliferation and differentiation of osteoblastic cells or inhibit osteoclastic activity, thus resulting in abnormal bone formation. Several candidate factors with critical roles as regulators of bone and prostate cell function, including IGFs, FGFs, TGFβ, BMPs, ET-1, and uPA, have been proposed as potential mediators for the development of osteoblastic metastases [Guise and Mundy, 1998]. Most of these are either secreted by prostate cancer cell lines or found in prostate cancer tissue. Growth factors present in the bone matrix could also facilitate proliferation of prostate cancer cells. The destruction of bone, which occurs as a result of increased tumor growth, allows for the release of growth factors stored in the bone matrix [Roodman, 2003].

In the current study, we investigated the role of IGF-I in the effects of LNCaP prostate cancer cell conditioned media (CM) on MC3T3-E1 osteoblastic cells. Our studies show that LNCaP prostate cancer cell CM have stimulatory effects on cell proliferation (^{3}H-thymidine incorporation) and protein synthesis (^{14}C-proline incorporation) in MC3T3-E1 osteoblastic cells that are mediated through the MAP kinase signaling pathway. The effects of the CM were similar to those of IGF-I and inhibition of IGF-I action decreased the stimulatory effects of the CM treatment on the MC3T3-E1 cells, suggesting that IGF-I is an essential factor for these stimulatory effects.

MATERIALS AND METHODS

Cell Culture

MC3T3-E1 mouse osteoblastic cells (Riken Cell Bank, Japan) were grown and maintained in α-MEM supplemented with 5% heat-inactivated fetal bovine serum (HI-FBS) and 50 μg/ml gentamycin. LNCaP prostate carcinoma cells (ATCC, Rockville, MD) were maintained in RPMI 1640 medium supplemented with 10% HI-FBS and 100 U/ml penicillin + 100 μg/ml streptomycin. Both cell lines were maintained at 37°C with a 95% O_2/5% CO_2 atmosphere in a humidified incubator. Tissue culture reagents were purchased from Gibco-Invitrogen (Carlsbad, CA).

CM Preparation

Serum-free CM were collected from the LNCaP cells at confluence. Briefly, LNCaP cells were seeded in Primaria coated T-75 flasks (BD, Bedford, MA) at a cell density of 2×10^6 cells/flask. After 7 days of culture, cells reached confluence. The LNCaP monolayer was gently washed with sterile phosphate buffered saline (PBS) and serum-free RPMI; 15-ml serum-free RPMI 1640 medium was added to the flask and the cells were incubated for 24 h at 37°C. After 24 h, CM were removed and centrifuged for 20 min at 4°C to remove cell debris. Aliquots of CM were stored at −20°C. Concentrations of CM ranging from 1/3X–1/36X were used in the experiments. Cell counts for each flask of LNCaP CM were in the range of 1.13–1.2 million cells/ml of 1X CM.

Measurement of Cell Proliferation and Protein Synthesis in Osteoblasts

Mitogenic activity was determined by measuring ^{3}H-thymidine (TdR) incorporation, and protein synthesis was assessed by ^{14}C-proline incorporation. The osteoblast cells were seeded at an initial density of 1×10^5 cells/well in a 24-well Primaria coated plate (BD). Cells were allowed to attach for 24 h and then serum starved overnight in RPMI 1640 supplemented with 100 U/ml penicillin + 100 μg/ml streptomycin. The cells were treated for 24 h in RPMI 1640 medium without serum and supplemented with 100 U/ml penicillin + 100 μg/ml streptomycin. At the time of treatment, cells had reached 80–90% confluence. For the last 2 h of the culture, osteoblast cells were dual-labeled with ^{3}H-thymidine (0.5 μCi/ml) and ^{14}C-proline (0.05 μCi/ml). The cells were washed sequentially with ice-cold PBS (two times), 10% trichloroacetic acid (TCA) (two times), 95% ethanol (one time), and then solubilized in 0.5 N NaOH. Incorporation of the radiolabel was measured by liquid scintillation counting. Data are expressed as percent of mean control.

Western Blotting Analysis

MC3T3-E1 cells were seeded in 10-cm tissue culture dishes (VWR, West Chester, PA) in α-MEM supplemented with 5% HI-FBS and gentamycin. Following 2–3 days of culture, cells reached initial confluence and were serum starved overnight in RPMI 1640 medium supplemented with 100 U/ml penicillin + 100 μg/ml streptomycin. Treatment with IGF-I was for 10 min and treatments with CM or inhibitors were for 10 min or 24 h. Cells were preincubated with inhibitors for 1 h prior to treatment. Reactions were stopped by removing treatment media and washing cells with 4 ml of ice-cold PBS. Whole cell lysates were prepared from treated cells as previously described [Sanders and Stern, 1996]. An aliquot of the lysate sample was used for protein determination using the Lowry method [Lowry et al., 1951]. For immunoblotting, cellular extracts were fractionated by sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS–PAGE). Proteins were transferred to nitrocellulose membranes, and activated MAP kinase was detected with phospho-p42/44 (1:1,000), total p42-44 (1:1,000), phospho-p38 (1:1,000) antibodies (Cell Signaling Technologies, Beverly, MA). Anti-rabbit secondary antibodies were used for all p42/44 MAP kinase primary antibodies (Amersham, Piscataway, NJ) and anti-mouse secondary antibodies were used for phospho-p38 MAP kinase primary antibodies (Amersham). Immune complexes were visualized using Enhanced Chemiluminescence (Amersham). Densitometric analysis was carried out with the Scion Image Analysis program.

Statistical Analysis

Statistical analysis was carried out using Graph Pad Prism software (version 3.0). Significance of results was determined by Analysis of Variance and Newman–Keuls post test or Student's *t*-test as appropriate.

RESULTS

Diluted LNCaP CM Stimulate Cell Proliferation and Protein Synthesis in MC3T3-E1 Osteoblasts

As an in vitro model to study prostate cancer cell effects on bone, mouse osteoblast MC3T3-E1 cells were treated with serum-free CM from the LNCaP prostate cancer cell line. CM effects on cell proliferation and protein synthesis were measured by ^{3}H-thymidine incorporation and ^{14}C-proline incorporation, respectively. Undiluted (1X) CM from confluent LNCaP cells inhibited cell proliferation and protein synthesis (data not shown). However, diluted CM (1/3X–1/36X) had biphasic stimulatory effects on the MC3T3-E1 cells, a pattern that was observed in multiple experiments. Results from a representative experiment are shown in Figure 1. The greatest stimulatory responses were obtained with a 1/18X dilution of CM. Approximately twofold stimulation of thymidine and proline incorporation was elicited with CM treatment. In MTT cell viability assays, 1/3X, 1/18X, and 1/36X CM treatment

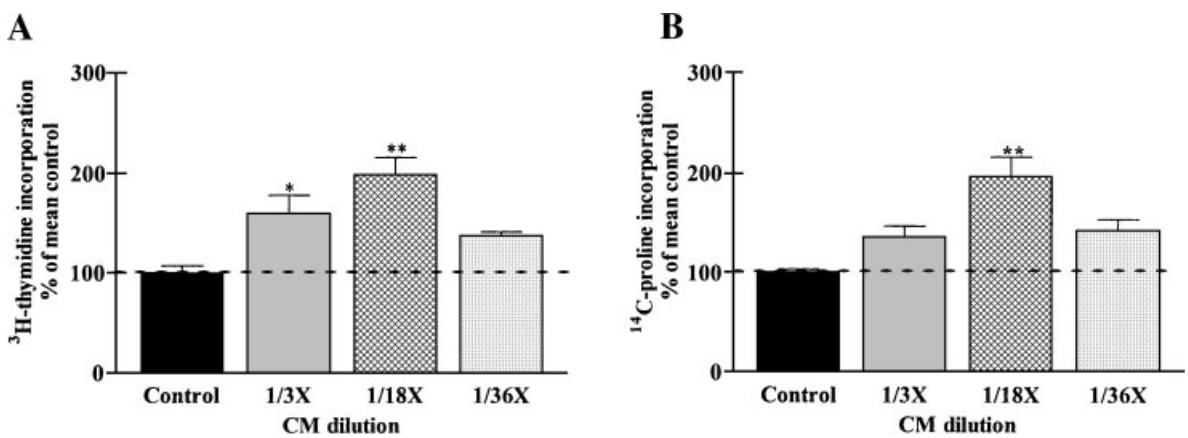


Fig. 1. Diluted LNCaP CM stimulate cell proliferation (^{3}H-thymidine incorporation) and protein synthesis (^{14}C-proline incorporation) in MC3T3-E1 cells. MC3T3-E1 osteoblastic cells were treated with LNCaP CM (1/3X–1/36X) for 24 h. Data are from a single representative experiment. **A**: ^{3}H-thymidine incorporation; (**B**) ^{14}C-proline incorporation. Values are means and standard errors of responses from four cultures. Control mean dpm: thymidine = 4,939 dpm/ml; proline = 485 dpm/ml. $^{*}P < 0.05$, $^{**}P < 0.01$ vs. control.

did not significantly affect cell viability, whereas 1X CM decreased cell viability (data not shown). Further experiments utilized a 1/18X dilution of LNCaP CM.

MAP Kinase Mediates the Proliferative Response of MC3T3-E1 Cells to LNCaP CM

Since MAP kinase has been found to be a signaling pathway for proliferative stimuli in osteoblastic cells [Hipskind and Bilbe, 1998], the effects of LNCaP CM on MAP kinase were determined. Treatment of MC3T3-E1 cells with LNCaP CM (1/18X) for 10 min increased the expression of phosphorylated p42/44 MAP kinase (Fig. 2A). MAP kinase is activated by the upstream kinase MAP kinase kinase (MEK). A MEK inhibitor, PD98059 (Calbiochem, San Diego, CA) (5 μM) which blocked MAP kinase activation (Fig. 2B), inhibited the stimulatory effect of LNCaP CM on phosphorylated p42/44 MAP kinase. PD98059 (5 μM) also significantly decreased the stimulatory effect of LNCaP CM on cell proliferation and protein synthesis in the MC3T3-E1 cells (Fig. 3A,B). PD98059 (5 μM) elicited a 50% inhibition of the CM stimulated response with no significant effect on control levels of thymidine and proline incorporation. The stimulation of p44 MAP kinase by LNCaP CM and the inhibitory effect of PD98059 on phosphorylated p42/44 were sustained and still observed at 24 h (Fig. 3C). In contrast, LNCaP CM and the MEK inhibitor failed to affect phosphorylated p38 MAP kinase in the MC3T3-E1 cells at 24 h (Fig. 3C). These data indicate that the p42/44 MAP kinase pathway is selectively involved in the effects of LNCaP CM to stimulate cell proliferation and protein synthesis in MC3T3-E1 osteoblastic

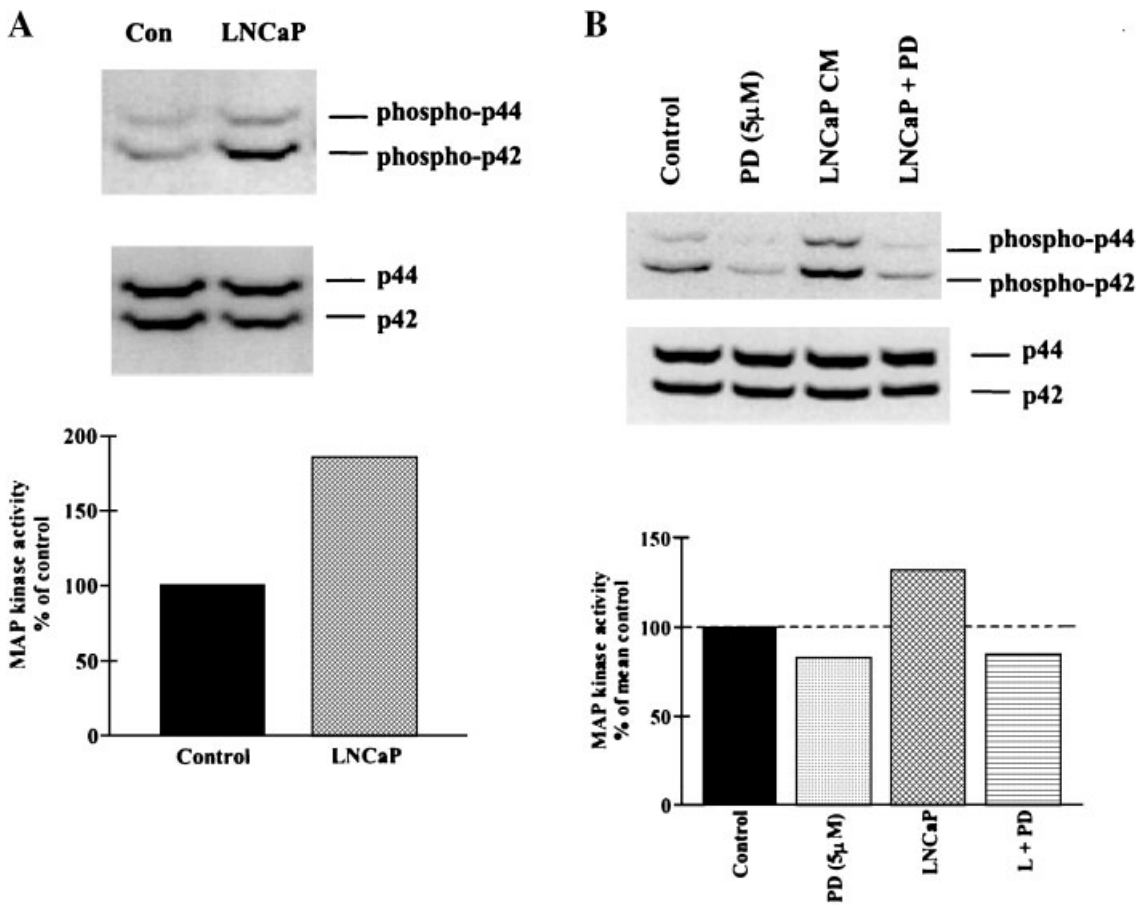


Fig. 2. LNCaP CM (1/18X) stimulate p42/44 MAP kinase activity. MC3T3-E1 osteoblastic cells were treated with CM for 10 min. MAP kinase activity was examined by Western blotting with phospho-specific p42/44 MAP kinase antibodies. **A**: LNCaP CM (1/18X) treatment; (**B**) LNCaP CM (1/18X) in combination with PD98059 (5 μM). MC3T3-E1 cells were preincubated with inhibitor for 1 h prior to treatment. Western blots were quantitated by densitometry. Phosphorylated p42/44 was normalized to total p42/44.

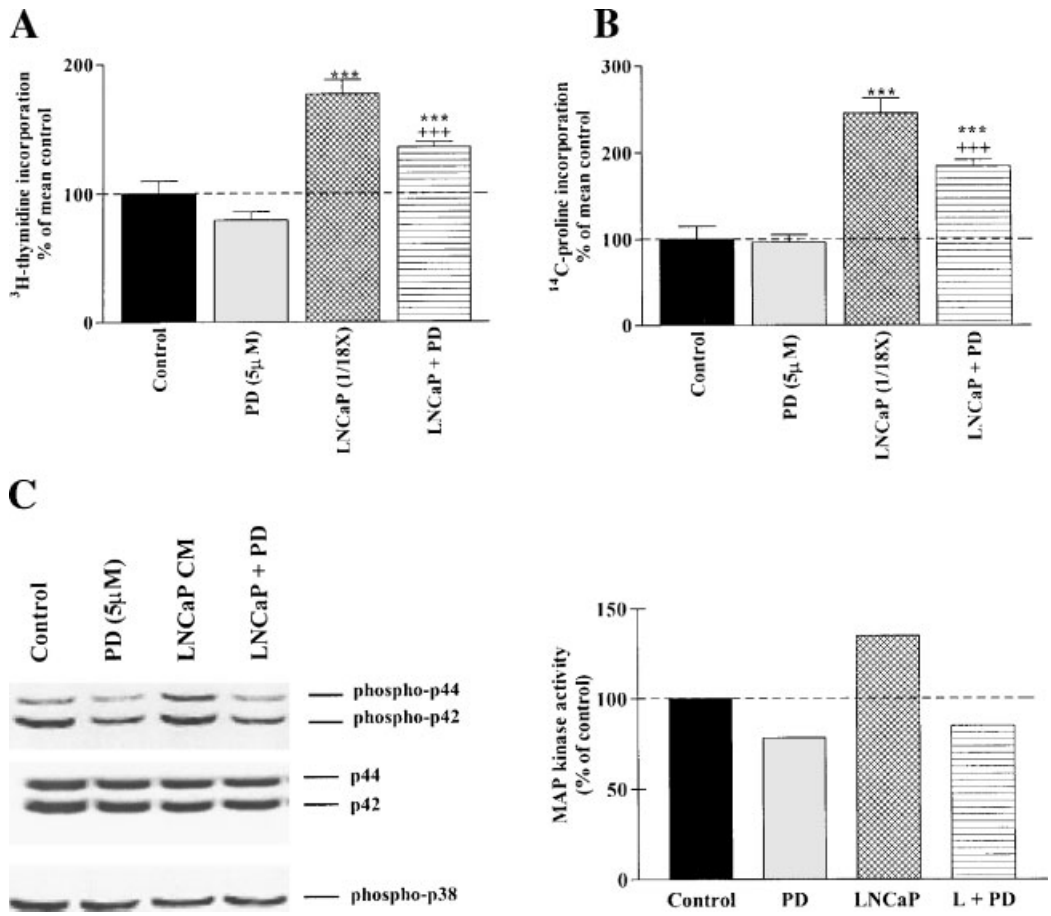


Fig. 3. LNCaP CM stimulate cell proliferation (^{3}H-thymidine incorporation) and protein synthesis (^{14}C-proline incorporation) in MC3T3-E1 cells through a MAP kinase dependent pathway. **A**: ^{3}H-thymidine incorporation; (**B**) ^{14}C-proline incorporation. MC3T3-E1 osteoblastic cells were treated with LNCaP CM (1/18X) in combination with PD98059 (5 μM) for 24 h. Values are means and standard errors of responses from four cultures. Control mean dpm: thymidine = 34,724 dpm/ml; proline = 1,076 dpm/ml. **C**: Western blot analysis of MAP kinase activity, phospho-p42/44, total p42/44, and phospho-p38. MC3T3-E1 cells were co-incubated with PD98059 (5 μM) and LNCaP CM for 24 h. Western blots were quantitated by densitometry. Phosphorylated p42/44 was normalized to total p42/44. $^{***}P < 0.001$ vs. control; $^{+++}P < 0.001$ vs. LNCaP (1/18X).

cells. The data also confirm the selectivity of the MEK inhibitor PD98059 in this system.

Cell Proliferation and Protein Synthesis Effects of IGF-I on MC3T3-E1 Cells Are Mediated Through MAP Kinase

Insulin-like growth factor (IGF-I) (BioSource, Camarillo, CA) is a bone anabolic factor that is also produced by prostate cancer cells [Pietrzkowski et al., 1992]. IGF (1–100 nM) stimulated cell proliferation and protein synthesis in the MC3T3-E1 osteoblasts in a dose-dependent manner (Fig. 4A,B). Significant effects were elicited by 1 nM IGF-I and the increases in both thymidine and proline incorporation were maximal at 10 nM, a concentration that produced a greater than twofold increase. Lower concentrations of IGF-I (<1 nM) did not elicit measurable stimulation of either cell proliferation or protein synthesis (data not shown). Ten-minute treatment of the MC3T3-E1 cells with IGF-I (10 nM) also stimulated MAP kinase activity (Fig. 5A). Effects on MAP kinase were elicited by concentrations of IGF-I as low as 0.1 nM and were dose-dependent at higher concentrations (Fig. 5B). PD98059 (3 μM) decreased IGF-I (10 μM) stimulation of thymidine and proline incorporation (Fig. 6A,B).

Inhibitors of IGF-I Action Decrease LNCaP CM Responses in MC3T3-E1 Cells

To determine the involvement of IGF-I in the observed stimulatory effects of LNCaP CM on osteoblasts, inhibitors of IGF-I action, αIR3 (Calbiochem, San Diego, CA), JB1 (Peninsula

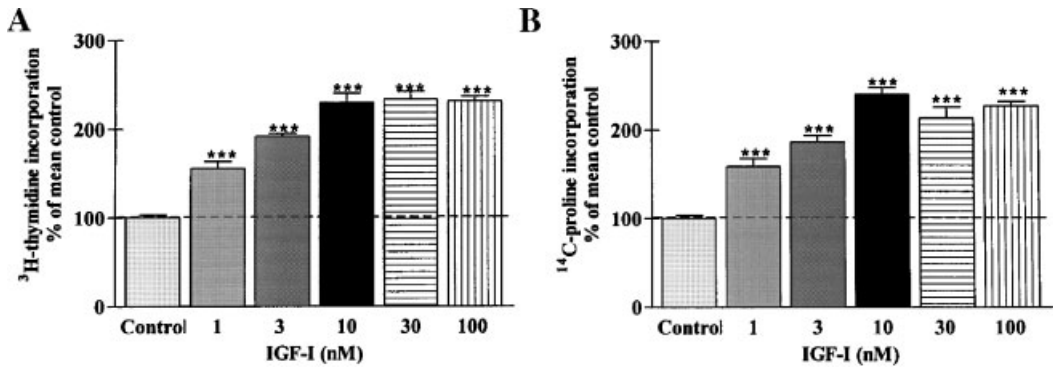


Fig. 4. IGF-I dose-dependently stimulates cell proliferation (^{3}H-thymidine incorporation) and protein synthesis (^{14}C-proline incorporation) in MC3T3-E1 cells. MC3T3-E1 osteoblastic cells were treated with IGF-I (1–100 nM) for 24 h. **A**: ^{3}H-thymidine incorporation; (**B**) ^{14}C-proline incorporation. Values are means and standard errors of responses from four cultures. Control dpm: thymidine = 17,407 dpm/ml; proline = 1,131 dpm/ml. ***$P < 0.001$ vs. control.

Labs, San Carlos, CA), and IGFBP3 (BioSource, Camarillo, CA) were used.

αIR3 (1 μg/ml), a monoclonal blocking antibody specific for the IGF-I receptor [Kull et al., 1983], completely blocked LNCaP CM stimulation of thymidine and proline incorporation in MC3T3-E1 cells (Fig. 7A,B). αIR3 (1 μg/ml) partially decreased IGF-I (10 nM) stimulation of thymidine and proline incorporation (Fig. 7C,D).

JB1, a peptide analog of IGF-I, inhibits IGF-I activity [Pietrzkowski et al., 1992]. JB1 (4 μg/ml) partially decreased LNCaP CM

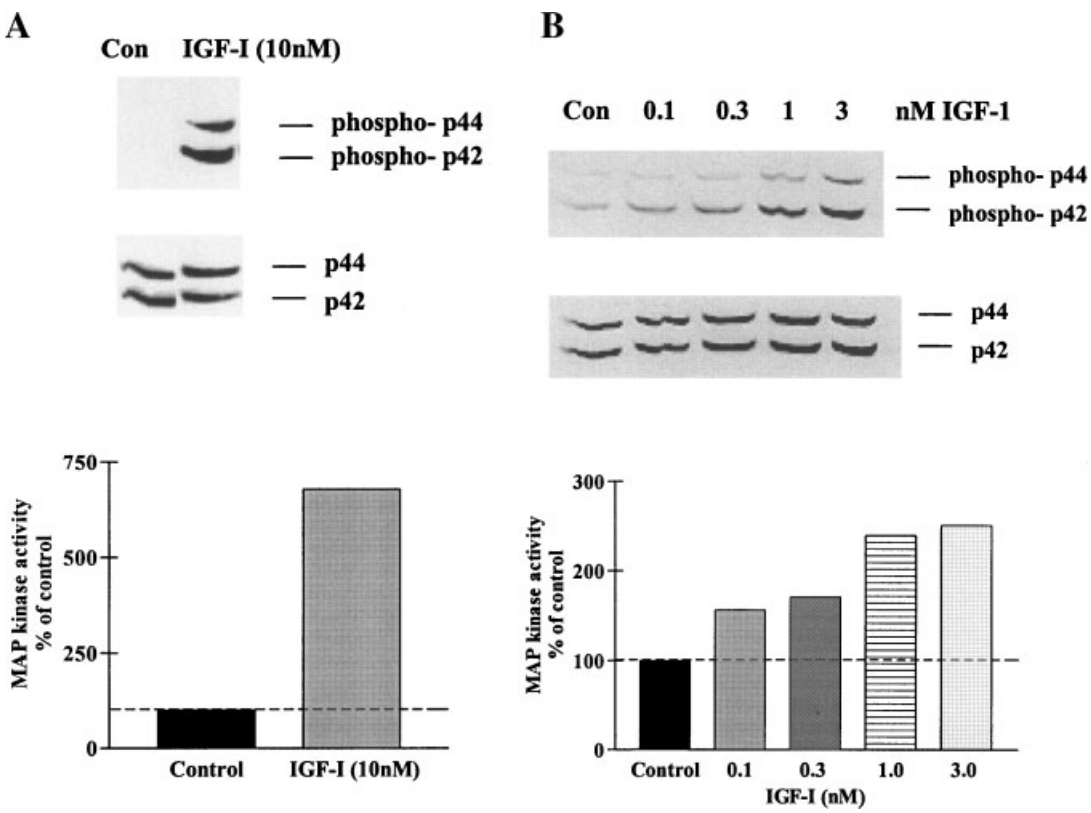


Fig. 5. IGF-I stimulates p42/44 MAP kinase activity in MC3T3-E1 cells. MC3T3-E1 osteoblastic cells were treated with IGF-I (0.1–10 nM) for 10 min. MAP kinase activity was examined by Western blotting with phospho-specific p42/44 MAP kinase antibodies. **A**: IGF-I (10 nM); (**B**) IGF-I (0.1–3 nM). Western blots were quantitated by densitometry. Phosphorylated p42/44 was normalized to total p42/44.

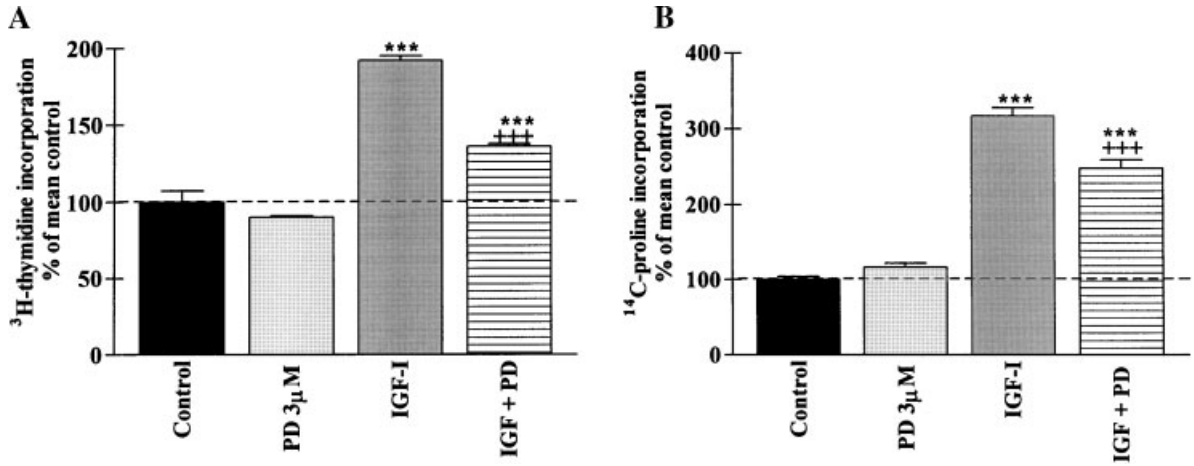


Fig. 6. IGF-I stimulates cell proliferation (^{3}H-thymidine incorporation) and protein synthesis (^{14}C-proline incorporation) in MC3T3-E1 cells, and the effects are prevented by the MEK inhibitor PD98059 (PD). **A**: ^{3}H-thymidine incorporation; (**B**) ^{14}C-proline incorporation. MC3T3-E1 osteoblastic cells were treated with IGF-I (10 nM) in combination with PD98059 (3 μM) for 24 h. Values are means and standard errors of responses from four cultures. Control dpm: thymidine = 22,882 dpm/ml; proline = 1,084 dpm/ml. $^{***}P < 0.001$ vs. control; $^{+++}P < 0.001$ vs. IGF-I.

stimulation of cell proliferation and protein synthesis in the MC3T3-E1 cells (Fig. 8A,B). IGF-I stimulation of cell proliferation and protein synthesis was also decreased by JB1 (Fig. 8C,D).

The IGF-binding protein IGFBP3 (BP3) can inhibit effects of IGF-I in vitro [Kawaguchi et al., 1994; Grimberg and Cohen, 2000]. Effects of LNCaP CM on thymidine and proline incorporation in MC3T3-E1 cells were decreased significantly by IGFBP3 (0.3 nM) (Fig. 9A,B). Higher concentrations of IGFBP3 inhibited control responses (data not shown). Since >10X excess of IGFBP3 is required to inhibit IGF [Schmid et al., 1991; Kawaguchi et al., 1994], and IGF concentrations of 1 nM or greater were required to show stimulatory effects (see above), it was not feasible to demonstrate inhibition of IGF-I with 0.3 nM IGFBP3 in 24-h cultures.

In short-term experiments, IGFBP3 (3 nM) and JB1 (8 μg/ml) decreased LNCaP CM stimulation of MAP kinase activity at 10 min (Fig. 10). IGFBP3 had greater inhibitory effects on LNCaP CM stimulation of phosphorylated p42/44 MAP kinase than JB1. Both IGFBP3 and JB1 elicited slight inhibitory effects on basal MAP kinase activity.

The experiments utilizing the IGF-I inhibitors (αIR3, JB1, IGFBP3) indicated that IGF-I receptor stimulation is essential for stimulatory effects of LNCaP CM on cell proliferation and protein synthesis.

DISCUSSION

The development of osteoblastic metastases is an established consequence of metastatic prostate cancer. The formation of these metastases may be promoted by growth factors either from the carcinoma cells or growth factors present within the bone matrix. The mediators and signaling pathways involved in the development of skeletal metastases have not been clearly defined. The current studies show that CM from LNCaP cells stimulate thymidine and proline incorporation by MC3T3-E1 osteoblastic cells and also activate the MAP kinase pathway in the MC3T3-E1 cells. A MEK inhibitor prevented the effects on thymidine and proline incorporation, demonstrating that the MAP kinase pathway is critical for the CM-induced stimulation of osteoblast cell proliferation and protein synthesis. Our findings also indicate that the stimulatory response of the osteoblastic cells to LNCaP CM requires IGF-I and IGF-I receptor action. The inhibitors of IGF-I action, αIR3, JB1, and IGFBP3, significantly decreased the stimulatory effects of CM on cell proliferation, protein synthesis, and MAP kinase activity.

Our in vitro culture system utilized LNCaP prostate cancer cell CM and MC3T3-E1 osteoblastic cells. LNCaP cells were originally derived from a human lymph node metastasis [Horoszewicz et al., 1980]. In certain animal

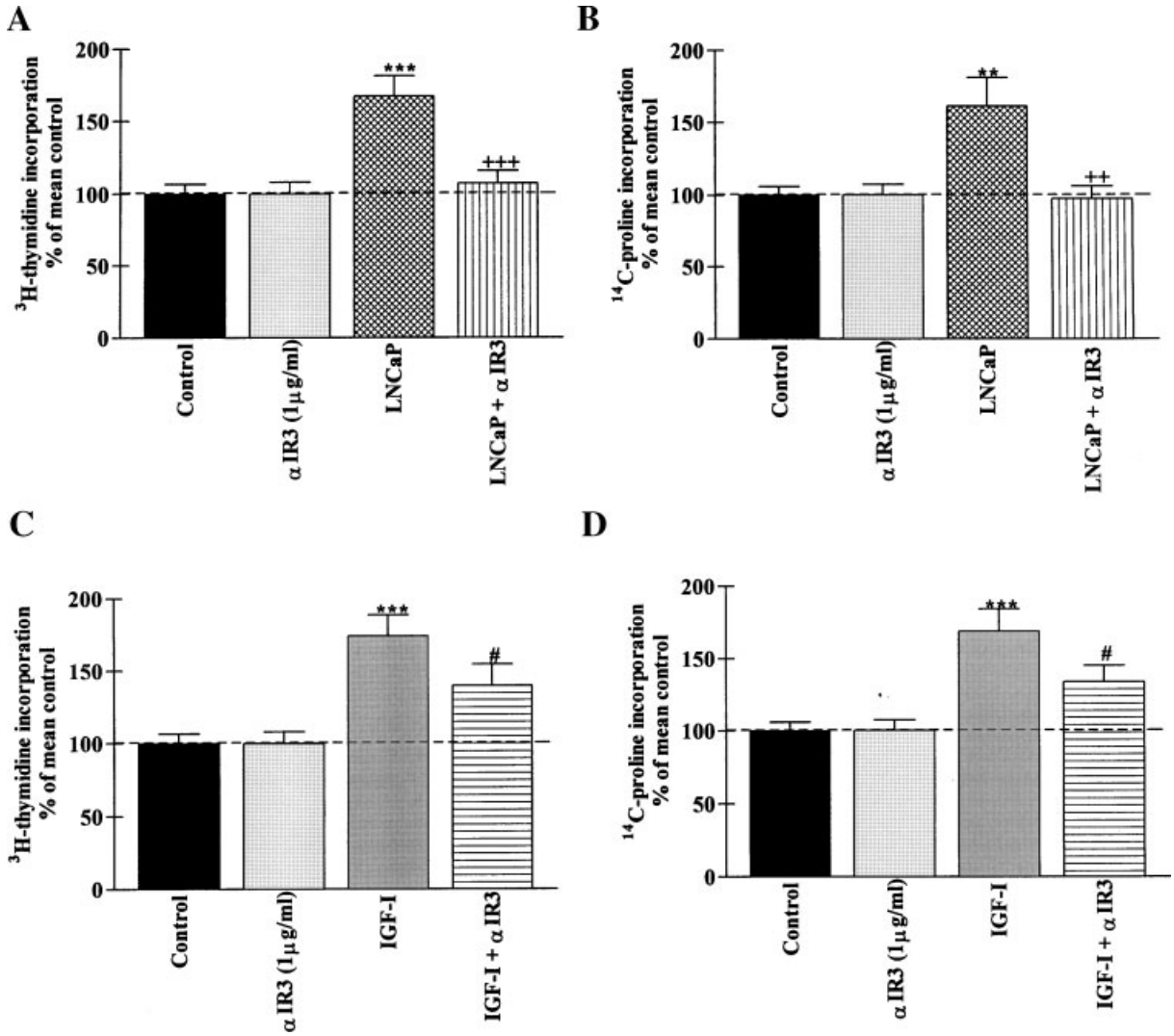


Fig. 7. LNCaP CM stimulation of cell proliferation (^{3}H-thymidine incorporation) and protein synthesis (^{14}C-proline incorporation) in the MC3T3-E1 cells is blocked by αIR3, a monoclonal blocking antibody specific for the IGF-I receptor. MC3T3-E1 osteoblastic cells were treated with LNCaP CM (1/18X) in combination with αIR3 (1 μg/ml) or IGF-I (10 nM) in combination with αIR3 (1 μg/ml). **A**,**C**: ^{3}H-thymidine incorporatio; (**B**,**D**) ^{14}C-proline incorporation. Values are means and standard errors of responses from eight cultures. Control dpm: thymidine = 41,586 dpm/ml; proline = 2,767 dpm/ml. $^{**}P < 0.01$, $^{***}P < 0.001$ vs. control; $^{\#}P < 0.05$ vs. IGF-I; $^{++}P < 0.01$, $^{+++}P < 0.001$ vs. LNCaP (1/18X).

model systems such as the SCID-hu model, LNCaP cells produce osteoblastic responses [Nemeth et al., 1999]. MC3T3-E1 cells are non-transformed cells with the capacity to differentiate into fully mature and mineralized osteoblasts [Sudo et al., 1983]. MC3T3-E1 cells also produce abundant levels of various bone growth factors including IGF-I [Amarnani et al., 1993; Thrailkill et al., 1995; Conover, 1996].

By utilizing serum-free CM, we were able to examine the effects of soluble factors produced by the LNCaP prostate cancer cells. As a method of standardizing CM, cell counts were determined in each flask of CM. Each milliliter of 1X CM represented approximately 1.13–1.2 million cells. Our studies showed biphasic effects on both cell proliferation and protein synthesis, in that a 1/18X dilution of LNCaP CM produced greater stimulatory responses than more concentrated media, and 1X CM significantly inhibited thymidine and proline incorporation (data not shown), indicating the presence of both inhibitory and stimulatory factors in the CM studies. Additionally, MTT cell viability assays indicated that undiluted LNCaP CM had inhibitory effects on cell viability (data not shown).

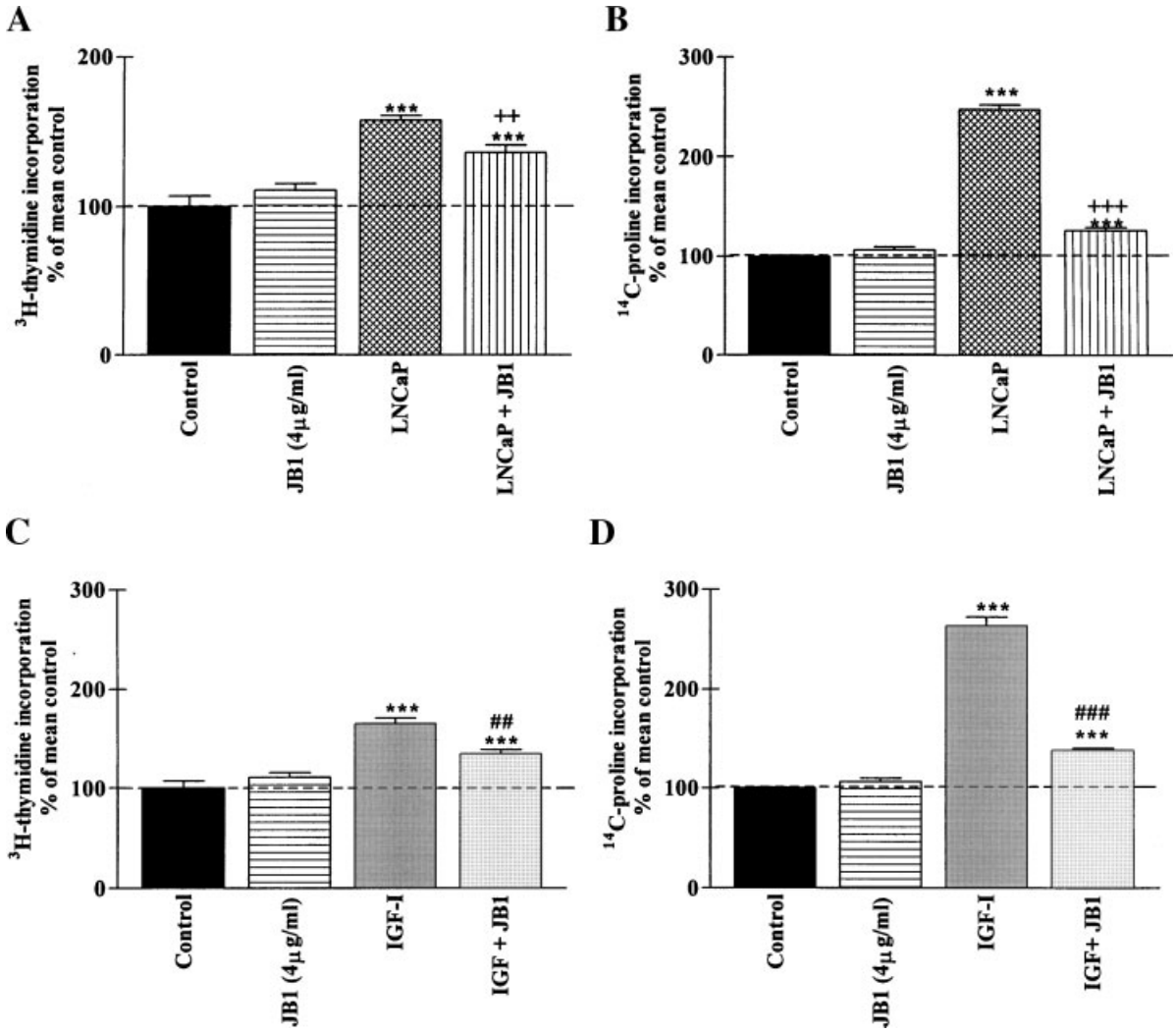


Fig. 8. LNCaP CM stimulation of cell proliferation (^{3}H-thymidine incorporation) and protein synthesis (^{14}C-proline incorporation) in the MC3T3-E1 cells is blocked by JB1, a peptide analog of IGF-I. MC3T3-E1 osteoblastic cells were treated with LNCaP CM (1/18X) in combination with JB1 (4 μg/ml) or IGF-I (3 nM) in combination with JB1 (4 μg/ml). **A**,**C**: ^{3}H-thymidine incorporation; (**B**,**D**) ^{14}C-proline incorporation. Values are means and standard errors of responses from four cultures. Control dpm: thymidine = 38,287 dpm/ml; proline = 2,551 dpm/ml. $^{***}P < 0.001$ vs. control; $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ vs. IGF-I; $^{++}P < 0.01$, $^{+++}P < 0.001$ vs. LNCaP (1/18X).

In order to verify the specificity of the LNCaP CM effects, preliminary studies were carried out utilizing CM isolated from the breast cancer cell line MDA-MB-231. Treatment of MC3T3-E1 cells with 1/3X, 1/9X, or 1/18X CM from the MDA-MB-231 cells, which were at approximately the same density as the LNCaP cells, failed to significantly stimulate cell proliferation or protein synthesis (data not shown). A 1X concentration of MDA-MB-231 CM was required to stimulate cell proliferation and protein synthesis (data not shown). These data suggest that the responses elicited by diluted LNCaP CM are specific for this cell line and do not represent a general response of osteoblast cells to epithelial cell CM. The findings are consistent with the effects of prostate cancer cell metastases to stimulate bone formation.

The parameters examined in this study included cell proliferation, protein synthesis, and MAP kinase activity. Cell proliferation and protein synthesis are measured by ^{3}H-thymidine and ^{14}C-proline incorporation, respectively. Proline incorporation functions as an indicator of collagen synthesis, a major component of the bone matrix, due to the high percentage (20%) of proline present in collagen. The MAP kinase pathway is commonly activated by growth factors and is involved in the regulation of cell proliferation. In osteoblasts, this is also

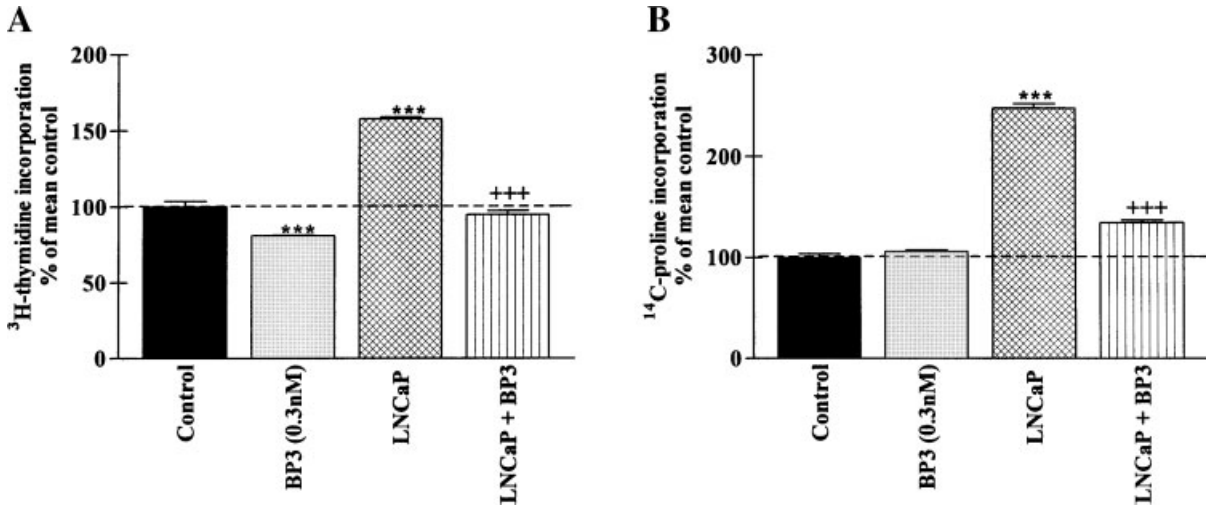
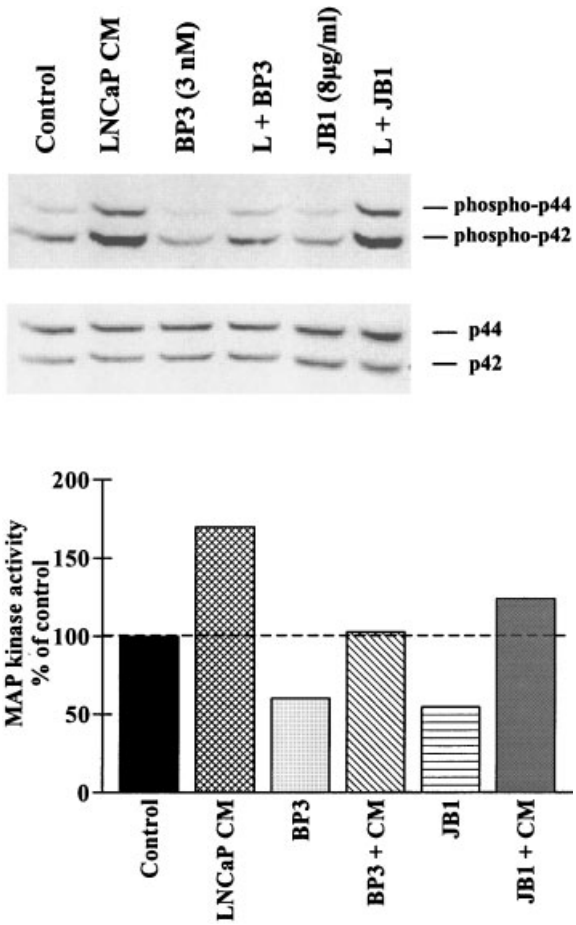


Fig. 9. LNCaP CM stimulation of cell proliferation (^{3}H-thymidine incorporation) and protein synthesis (^{14}C-proline incorporation) in the MC3T3-E1 cells is blocked by IGFBP3 (BP3), one of the IGF binding proteins with inhibitory properties. MC3T3-E1 osteoblastic cells were treated with LNCaP CM (1/18X) in combination with IGFBP3 (0.3 nM) for 24 h. **A**: ^{3}H-thymidine incorporation; (**B**) ^{14}C-proline incorporation. Values are means and standard errors of responses from four cultures. Control dpm: thymidine = 27,594 dpm/ml; proline = 1,073 dpm/ml. $^{***}P < 0.001$ vs. control; $^{+++}P < 0.001$ vs. LNCaP.



the case, with the MAP kinase cascade functioning as an important component in the signaling pathways involved in proliferation [Hipskind and Bilbe, 1998]. Growth factors, such as IGF-I, are also classic activators of the MAP kinase signaling pathway.

IGF-I is an established skeletal growth factor promoting both osteoblast proliferation and differentiated functions [Canalis, 1980; Canalis and Lian, 1988; McCarthy et al., 1989a, 2000; Linkhart et al., 1996; Chihara and Sugimoto, 1997]. The current studies compared the effects of IGF-I on cell proliferation and protein synthesis in MC3T3-E1 osteoblastic cells with those of LNCaP CM. IGF-I dose dependently stimulated cell proliferation and protein synthesis in the osteoblastic cells (Fig. 4). p42/44 MAP kinase was also increased in a dose-dependent manner (Fig. 5). The MEK inhibitor PD98059 significantly decreased the IGF-I-induced increase in cell proliferation and protein synthesis, whereas the p38 inhibitor SB203580 did not (data not shown), indicating that the p42/44 MAP kinase pathway is selectively involved in

Fig. 10. LNCaP CM stimulation of p42/44 MAP kinase activity in MC3T3-E1 cells is blocked by JB1 and IGFBP3 (BP3). MAP kinase activity was examined by Western blotting with phospho-specific p42/44 MAP kinase antibodies. MC3T3-E1 osteoblastic cells were pre-incubated with either JB1 (8 µg/ml) or IGFBP3 (3 nM) for 1 h prior to LNCaP CM (1/18X) treatment (10 min). Western blots were quantitated by densitometry. Phosphorylated p42/44 was normalized to total p42/44.

the observed effects of IGF-I in the MC3T3-E1 cells (Fig. 6).

Expression of IGF-I in osteoblastic cells can also be regulated and is critical for effects of other growth factors and hormones [Canalis et al., 1993]. PTH actions on rat bone are mediated through increased expression of local IGF-I [Canalis et al., 1989; McCarthy et al., 1989a; Pfeilschifter et al., 1995]. Triiodothyronine (T3) increases IGF-I in bone and osteoblastic cells [Lakatos et al., 1993; Huang et al., 2000], and αIR3 and JB1 decrease these effects of T3 on MC3T3-E1 osteoblastic cells [Huang et al., 2000].

IGF-I and various components of the IGF-I system have been proposed to be markers for prostate cancer progression. Serum levels of IGF-I in men with metastatic prostate cancer are significantly elevated compared to levels in men with non-metastatic cancer [Wolk et al., 1998]. Additionally, levels of IGFBP3 are reduced in men with metastatic prostate cancer resulting in a greater amount of bioactive IGF-I [Pollak et al., 1998]. The ratio of IGF-I/IGFBP may serve as an important indicator in determining prostate cancer risk level [Pollak et al., 1998]. Expression of the IGF-I receptor has also been shown to be upregulated in primary prostate cancer at the protein and mRNA level compared to benign prostatic epithelium [Hellawell et al., 2002].

Mitogenic and anti-apoptotic actions of IGF-I could promote the development of prostate cancer [Djavan et al., 2001]. In prostate stromal cells, IGF-I reduced camptothecin-induced apoptosis to control levels [Grant et al., 1998]. IGF significantly stimulated cell proliferation, DNA synthesis, and promoted chemotaxis in LNCaP, PC3, and DU145 prostate carcinoma cells [Ritchie et al., 1997]. IGF-I, in the tumor environment, can also promote effects of androgen to increase prostate specific antigen (PSA) production. In LNCaP cells, 50 ng/ml IGF-I elicits a fivefold increase in PSA levels [Culig et al., 1994]. In the tumor environment, PSA, which is also an IGFBP protease, can increase IGF-I bioactivity by cleaving IGFBP3 and preventing IGF-I from being sequestered in an inactive state [Pollak et al., 1998].

In order to determine the role of IGF-I in the LNCaP CM effects on MC3T3-E1 cells, three different inhibitors of IGF-I action were utilized, αIR3, JB1, and IGFBP3. αIR3 and JB1 both inhibit IGF-I at the receptor level and IGFBP3 functions by sequestering IGF-I and preventing activation of the receptor. αIR3 is a monoclonal antibody specific for the IGF-I receptor and prevents IGF binding to the IGF-I receptor. αIR3 has been shown to antagonize the growth-promoting effects of endogenous or exogenous IGF-I in various cell systems [Arteaga and Osborne, 1989; Baier et al., 1992; Lahm et al., 1994; Raile et al., 1994]. JB1 is a peptide analog of IGF-I designed against a molecular model of the IGF-I protein [Pietrzkowski et al., 1992]. In various systems, JB1 has been shown to inhibit IGF-I action by binding and preventing activation of the IGF-I receptor. JB1 can also inhibit growth of several cell types including prostate carcinoma cells and SV40 transformed cells [Pietrzkowski et al., 1992]. As previously described, IGFBP3 (BP3) is one of the IGFBPs that can have inhibitory effects on IGF-I action. An excess of IGFBP3 has been shown to inhibit the IGF-I stimulation of thymidine incorporation in fetal rat calvarial cultures [Kawaguchi et al., 1994] and in rat osteoblastic cells [Schmid et al., 1991]. Since αIR3, JB1, and IGFBP3 prevent IGF-I receptor activation, it is possible that they could also be inhibiting effects of IGF-II which is a weaker activator of the IGF-I receptor [McCarthy et al., 1989b; Centrella et al., 1990; Schmid, 1995]. IGFBP3 also has a greater affinity for IGF-I, compared to IGF-II [Grimberg and Cohen, 2000].

The current data from the three IGF-I inhibitors demonstrate that IGF-I has a critical role in the LNCaP CM effects on MC3T3-E1 cells. All three inhibitors decreased the LNCaP CM stimulation of cell proliferation, protein synthesis in the osteoblastic cells (Figs. 7–9). αIR3 and IGFBP3 almost completely inhibited the LNCaP-induced stimulation of thymidine and proline incorporation (Figs. 7 and 9), while JB1, at the concentration and time point tested, produced only a partial inhibition (Fig. 8). The stimulatory effects of IGF-I on thymidine and proline incorporation were also only partially inhibited by the 4 μg/ml JB1 (Fig. 8C,D). Basal responses in MC3T3-E1 cells were also more sensitive to IGFBP3 than to JB1. IGFBP3 (1 nM) treatment of MC3T3-E1 cells significantly decreased cell proliferation (data not shown), and concentrations as low as 0.3 nM had occasional inhibitory effects on cell proliferation (Fig. 9A). Basal protein synthesis was also decreased by 0.3 nM IGFBP3, although this inhibitory effect was not observed in all experiments.

The current data indicate that IGF-I is critical for the stimulatory effects of LNCaP CM on cell proliferation, protein synthesis, and MAP kinase activity in MC3T3-E1 cells. However, the source of the IGF-I for these effects is not clear. Previous studies, utilizing a sensitive assay, indicate that LNCaP cells do not make detectable amounts of IGF-I [Iwamura et al., 1993; Rubin et al., 2002]. An alternative possibility is that the LNCaP cell CM could be stimulating release of IGF-I from bone. Bone is an abundant source of IGF-I. As previously described, IGF-I is produced by bone cells and is stored in the bone matrix [Canalis and Lian, 1988; Canalis et al., 1988; Hock et al., 1989]. MC3T3-E1 cells produce IGF-I mRNA and protein. Studies by Amarnani et al. [1993] have shown that IGF-I produced by MC3T3-E1 cells can have autocrine effects, stimulating cellular proliferation on these same osteoblastic cells. Thus the MC3T3-E1 cells could have been the source of IGF-I in the present studies. Although IGF-I is clearly essential, it is conceivable that other factors present in the CM or produced by the bone cells could synergize with IGF-I and potentiate its effects to stimulate bone formation. The current studies also suggest that the IGF-I pathway could serve as a potential target for new therapies to treat osteoblastic metastases.

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