# Inhibition of Osteoblastic Cell Differentiation by Conditioned Medium Derived From the Human Prostatic Cancer Cell Line PC-3 In Vitro

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**Abstract** Human prostatic carcinoma frequently metastasizes to bone tissue and activates bone metabolism, especially bone formation, at the site of metastasis. It has been reported that an extract of prostatic carcinoma and conditioned medium (CM) of a human prostatic carcinoma cell line, PC-3, established from a bone metastastic lesion, stimulate osteoblastic cell proliferation. However, there is little information about the effect of PC-3 CM on the differentiation of osteoblastic cells. In this study, we investigated the effect of PC-3 CM on the differentiation of two types of osteoblastic cells, primary fetal rat calvaria (RC) cells containing many undifferentiated osteoprogenitor cells, and ROS 17/2.8, a well-differentiated rat osteosarcoma cell line. PC-3 CM inhibited bone nodule formation and the activity of alkaline phosphatase (ALPase), an osteoblastic marker enzyme, on days 7, 14, and 21 (RC cells) or 3, 6, and 9 (ROS 17/2.8 cells) in a dose-dependent manner (5–30% CM). However, the CM did not affect cell proliferation or cell viability. PC-3 CM was found to markedly block the gene expression of ALPase and osteocalcin (OCN) mRNAs but had no effect on the mRNA expression of osteopontin (OPN), the latter two being noncollagenous proteins related to bone matrix mineralization. These findings suggest that PC-3 CM contains a factor that inhibits osteoblastic cell differentiation and that this factor may be involved in the process of bone metastasis from prostatic carcinoma. J. Cell. Biochem. 67:248–256, 1997. © 1997 Wiley-Liss, Inc.

**Key words:** human prostatic cancer cell (PC-3); osteoblastic cell differentiation; bone nodule formation; alkaline phosphatase activity; osteocalcin; osteopontin

Prostatic carcinoma, commonly affecting men in late middle age, frequently metastasizes to bone similar to lung, breast, and kidney carcinomas, resulting in a poor prognosis [Chiarodo, 1991; Franks, 1973]. Carcinoma cells metastatic to bone adhere to bone tissue and resorb it, creating spaces for the growth of tumor nests. During this process, bone metabolism, including bone resorption and formation, is locally activated. Whereas lung and breast carcinomas invade bone tissue osteolytically, most prostatic carcinomas metastasize osteoblastically, resulting in new bone formation rather than degradation [Goltzman et al., 1992].

Previous studies have demonstrated that extracts of human prostatic carcinoma and hyperplastic tissue have mitogenic activity for osteoblastic cells [Koutsilieris et al., 1987; Jacobs and Lawson, 1980]. The PC-3 cell line, established from a human prostatic carcinoma that metastasized to bone tissue [Kaighn et al., 1979], has been widely used in studies of prostatic carcinoma metastasis because the cells retain the characteristics of prostatic carci-

Abbreviations: ALPase, alkaline phosphatase; BN, bone nodule; CM, conditioned medium; EGF, epidermal growth factor; FGF, fibroblast growth factor; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; OCN, osteocalcin; OPN, osteopontin; RC, rat calvaria; SDS, sodium dodecyl sulfate; TGF-β, transforming growth factor-β.

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noma that has metastasized to bone. Simpson et al. [1985] showed that conditioned medium collected from PC-3 cell cultures (PC-3 CM) contained a factor stimulating cell proliferation and the activity of alkaline phosphatase (ALPase), a marker enzyme of osteoblastic cell differentiation, in the rat osteoblastic osteosarcoma cell line UMR106. The factor present in PC-3 CM was indicated to be a $NH_2$-terminal fragment of urokinase-type plasminogen activator, the specific receptor for which is present in the human osteoblast-derived osteosarcoma cell line SAOS-2 and rat calvaria cells [Rabbani et al., 1990]. Moreover, Kimura et al. [1992] have histologically demonstrated that PC-3 CM stimulates the calcification of the human osteoblastic cell line Tak-10 in vitro. These reports suggest that PC-3 CM as well as prostatic carcinoma tissue contain a factor that stimulates the proliferation and differentiation of osteoblastic cells and that this might be associated with the osteoblastic metastasis of prostatic carcinoma cells.

Recently, Martínez et al. [1996] showed that PC-3 CM increased the proliferation of osteoblastic cells derived from fetal rat calvaria and decreased ALPase activity and cellular $Ca^{2+}$ accumulation, indicating the presence of an inhibitory factor of osteoblastic cell differentiation. Harrod et al. [1983] reported that PC-3 CM, which markedly increased DNA synthesis in osteoblastic cells, also had bone-resorbing activity. These effects of PC-3 CM on osteoblastic cell differentiation were opposite to those reported previously. Thus, details of the mechanism of the effect of PC-3 CM on osteoblastic cell differentiation in metastasized bone lesions are poorly understood.

In the present study, to elucidate the effect of PC-3 CM on osteoblastic cell differentiation during the process of prostatic carcinoma cell metastasis, we determined the number of bone nodules (BNs) and ALPase activity in two kinds of osteoblastic cells: primary fetal rat calvaria (RC) cells, which include many undifferentiated osteoprogenitor cells, differentiate into osteoblastic cells, and finally form mineralized BNs [Bellows et al., 1986; Bellows and Aubin, 1989], and ROS 17/2.8, a well-differentiated osteoblastic cell line. We also investigated the effect of PC-3 CM on the gene expression of ALPase, osteocalcin (OCN), and osteopontin (OPN), which are noncollagenous proteins associated with mineralization of the bone matrix and considered to be markers of mature osteoblasts differentiation [Hauschka et al., 1989; Canalis and Lian, 1988; Sodek et al., 1992; Kubota et al., 1989].

## MATERIALS AND METHODS

### Cell Cultures and Conditioned Medium Preparation

PC-3 cells ($4 \times 10^4$ cells/cm$^2$) were cultured in alpha modified Eagle's medium ($\alpha$-MEM), supplemented with 10% fetal calf serum (FCS) and antibiotics (100 units/ml penicillin G, 100 μg/ml streptomycin, 300 ng/ml amphotericin B) for 5 days, washed with PBS(−) three times, and further cultured in FCS-free, $\alpha$-MEM for 2 days. The collected medium was centrifuged to remove cell fragments, filtered with a 0.22 μm cellulose membrane, and stocked at −70°C before use. The CMs derived from Hela and two human salivary adenocarcinoma cell lines, HSG and HSY, were used for ALPase activity assay as negative controls.

RC cells were isolated from 21 day fetal rat calvaria by sequential collagenase digestion according to the method of Bellows et al. [1986], inoculated at a cell density of 3,000 cells/cm$^2$, and cultured in $\alpha$-MEM containing 10% FCS, 50 μg/ml ascorbic acid, and 2 mM $\beta$-glycerophosphate for 7–21 days. ROS 17/2.8 cells were seeded at a cell density of $2.8 \times 10^4$ cells/cm$^2$ and cultured in $\alpha$-MEM containing 10% FCS for 3–9 days.

### Assays of BN Formation, ALPase Activity, DNA Content, and Cell Viability

For the assay of BN formation, RC cells cultured for 21 days were fixed with 10% neutral buffered formalin and stained in situ by the von Kossa technique. The number of mineralized BNs was counted under a dissecting microscope. For assay of ALPase activity, RC and ROS 17/2.8 cells cultured in the presence of 0–30% PC-3 CM for 3–21 days were scraped into cold 50 mM Tris-HCl buffer (pH 7.4), sonicated, and centrifuged at 5,000 rpm for 10 min. ALPase activity in the supernatant was determined by the method of Lowry et al. [1954], with p-nitrophenyl phosphate as a substrate. Cellular DNA content was determined by the method of Hinegardner [1971]. Briefly, the cells cultured with PC-3 CM were washed with PBS(−), fixed with 100% ethanol for 5 min, and

dried in air, and then the cellular DNA content was determined fluorometrically with 3,5′-diamino benzoic acid. Cell viability was assayed using Alamar Blue™ solution (Alamar Biosciences Inc.).

### Northern Blot Analysis

Total cellular RNA was isolated by the acid guanidinium thiocyanate-phenol-chloroform method [Chomczynski and Sacchi, 1987]. Aliquots (5 μg) of RNA were separated by electrophoresis in 1% agarose gels containing 2 M formaldehyde and transferred to nylon membranes (Hybond-N+; Amersham Life Science, Buckinghamshire, UK). The cDNA probes for rat ALPase [Noda et al., 1987], rat OCN [Celeste et al., 1986], and mouse OPN (2ar) [Smith and Denhardt, 1987] were kindly supplied by Dr. G.A. Rodan (Merck Research Laboratories, West Point, PA), Dr. J.M. Wozney (Genetics Institute, Cambridge, MA), and Dr. D.T. Denhardt (Rutgers University, Piscataway, NJ), respectively. The cDNA probes were labeled with [$\alpha$-$^{32}$P]dCTP using a random primer DNA labeling kit (TAKARA, Kyoto, Japan). Prehybridization was performed for more than 4 h at 42°C in 50% formamide, 5× SSPE, 5× Denhardt's solution, 0.5% SDS, and 200 μg/ml salmon sperm DNA, and hybridization was done overnight at the same temperature with the appropriate probes ($1 \times 10^6$ dpm/ml buffer). The membranes were washed in 2× SSPE–0.1% SDS at 42°C and exposed to X-ray films at −70°C. The extent of hybridization was determined by densitometric scanning of the autoradiograms. The levels of ALPase, OCN, and OPN mRNA were normalized to that of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA.

## RESULTS

### Effect of PC-3 CM on BN Formation and ALPase Activity in Osteoblastic Cells

The BNs formed in RC cell cultures treated with PC-3 CM (0–30%) were delineated as dark dots, as shown in Figure 1A. PC-3 CM decreased BN formation in a dose-dependent manner (Fig. 1B). At 5% CM, the number of BNs diminished to 38% of the control (0% CM) and almost disappeared at more than 20% CM.

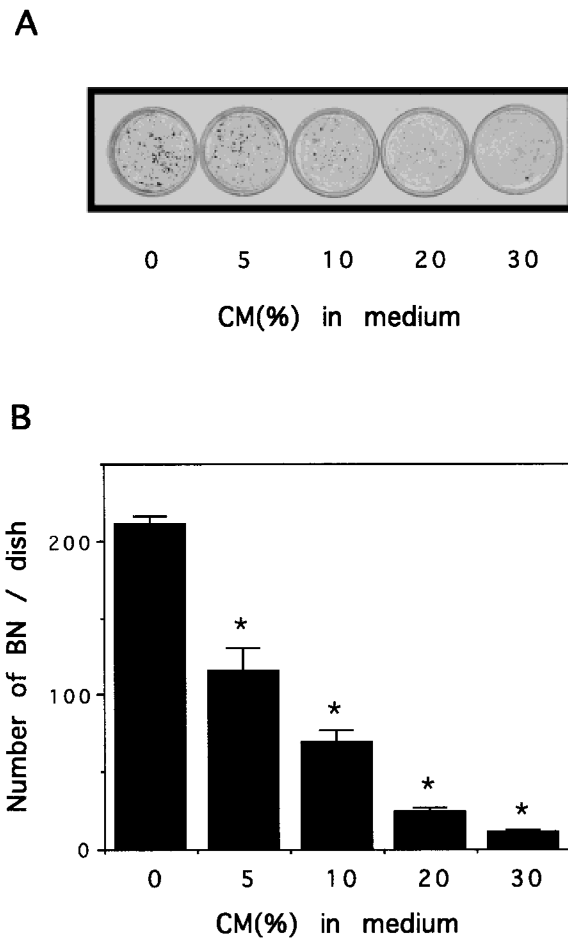


**Fig. 1.** Effect of PC-3 CM on BN formation by RC cells. RC cells ($3 \times 10^4$ cells) were inoculated in 35 mm dishes and cultured in medium for RC cells in the absence or presence of 5–30% PC-3 CM from the next day for 21 days. **A:** The BNs formed were stained by the von Kossa technique. **B:** The stained, mineralized BNs were counted under a dissecting microscope. Values are means ± S.E. for triplicate samples in three separate experiments. *Significantly different from 0% control ($P < 0.01$).

We then determined ALPase activity in two kinds of osteoblastic cells incubated with PC-3 CM. In RC cells containing undifferentiated osteoprogenitor cells, 20% PC-3 CM significantly decreased the enzyme activity to 33%, 24%, and 22% of the control level on days 7, 14, and 21, respectively (Fig. 2A). When RC cells were cultured with PC-3 CM at concentrations ranging from 5–30% for 14 days, ALPase activity decreased from 45–32% of the control in a dose-dependent manner (Fig. 2B). In ROS 17/2.8, a well-differentiated osteoblastic cell line, the CM induced significant inhibition of ALPase activity to 58%, 36%, and 46% of the control on days 3, 6, and 9, respectively (Fig. 2C), and dose-dependent decreases in the enzyme activity were observed at 0–30% CM to the same degree as that in RC cells (Fig. 2D). On the other hand, CMs derived from Hela and two human salivary adenocarcinoma cell lines,

HSG and HSY, had no significant effect on ALPase activity in ROS 17/2.8 cells (data not shown), suggesting that the CM of PC-3 cells specifically inhibited ALPase activity in the two osteoblastic cell lines.

## Effect of PC-3 CM on Osteoblastic Cell Proliferation

The effect of PC-3 CM on the proliferation of RC and ROS 17/2.8 cells was investigated by determining their DNA content. As shown in Figure 3A, 20% PC-3 CM did not affect the DNA content of RC cells on days 7, 14, and 21 in spite of exhibiting a marked inhibitory effect on ALPase activity (Fig. 2A). Similarly, there was no significant change in the DNA content of ROS 17/2.8 cells after treatment with 20% PC-3 CM on days 3, 6, and 9 (Fig. 3B). As shown in Figure 3C, no significant reduction of DNA content was observed at a CM concentration of less than 30%, and PC-3 CM had no effect on cell viability (5% CM: 93%; 10% CM: 94%; 20% CM: 94%; 30% CM: 92%). Thus, PC-3 CM did not induce any inhibitory effect on the proliferation of either of the osteoblastic cell lines.

## Effect of PC-3 CM on ALPase, OCN, and OPN mRNA Expression

The bands of OCN, OPN, and ALPase mRNA isolated from ROS 17/2.8 cells cultured with 0–30% PC-3 CM for 6 days are shown in Figure 4. When densities of the expressed bands were compared with those of mRNA derived from control cultures (0% CM), which was estimated as 1.0 by densitometric scanning, the bands of ALPase mRNA showed values of 0.5, 0.4, 0.3,

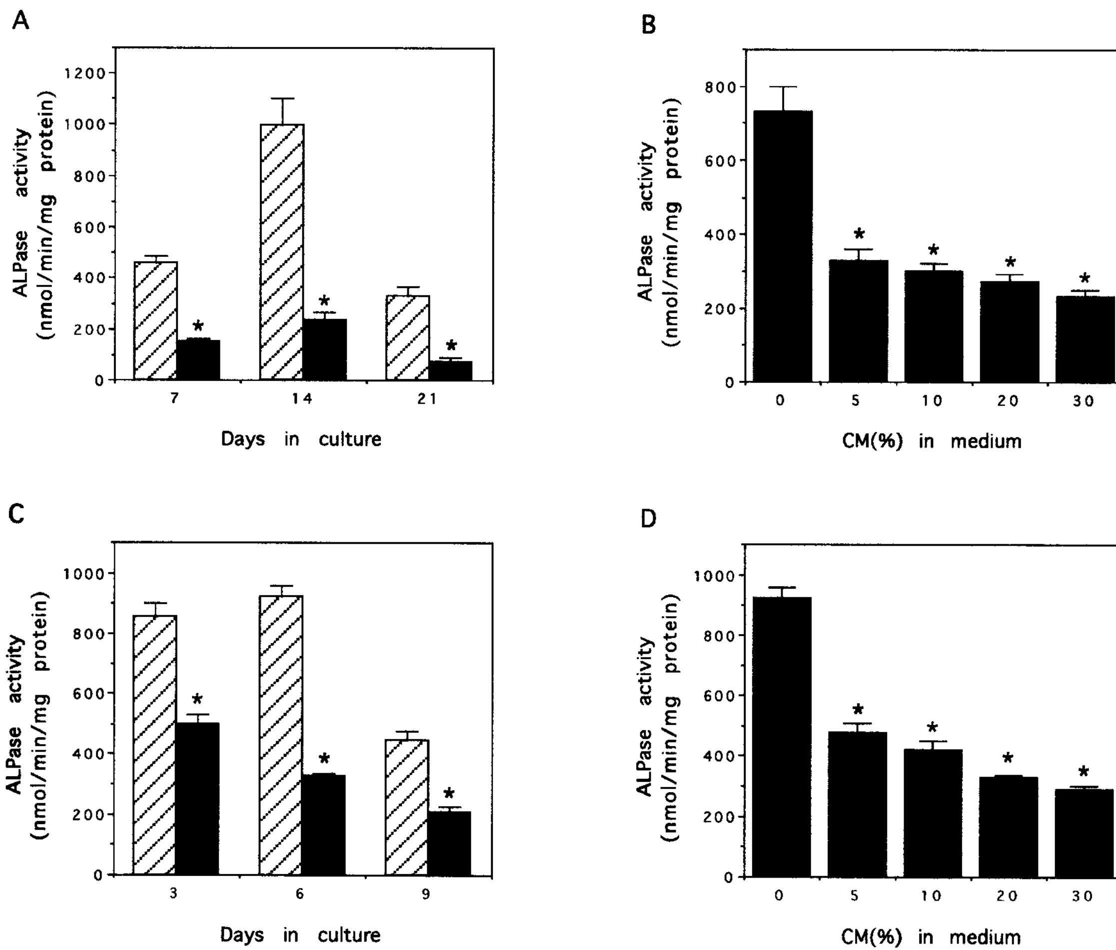


**Fig. 2.** Effect of PC-3 CM on ALPase activity in RC and ROS 17/2.8 cells. RC and ROS 17/2.8 cells were cultured in the indicated culture medium, as described in Materials and Methods. ALPase activity in RC (**A**) and ROS 17/2.8 (**C**) cells cultured with (solid column) or without (hatched column) 20% PC-3 CM was determined on the indicated days. RC (**B**) or ROS 17/2.8 (**D**) cells were cultured with 0–30% CM for 14 or 16 days, respectively, and the enzyme activity was determined. Values are means ± S.E. for quadruplicate samples. *Significantly different from 0% control ($P < 0.01$).

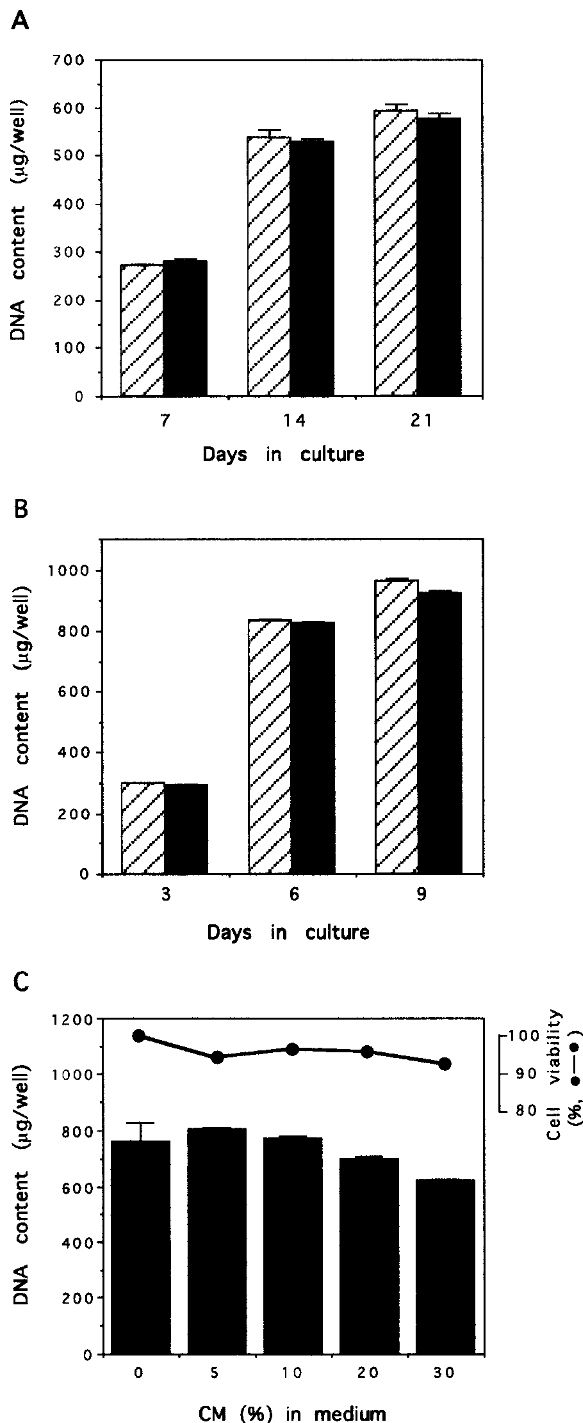


**Fig. 3.** Effect of PC-3 CM on DNA content and viability of RC and ROS 17/2.8 cells. RC (**A**) and ROS 17/2.8 (**B**) cells were cultured with (solid column) or without (hatched column) 20% PC-3 CM, and the DNA content was measured on days 7, 14, and 21. **C:** DNA content of ROS 17/2.8 cells cultured with 0–30% CM was determined on day 6, and their viability also was assayed. Values are means ± S.E. for quadruplicate samples. The viability of the control cell culture (0% CM) was assigned as 100%.

and 0.2 in 5%, 10%, 20%, and 30% CM, respectively, indicating dose-dependent inhibition by PC-3 CM. This inhibitory effect was very similar to that on ALPase activity. The CM completely blocked OCN mRNA expression (0% CM: 1.0; >5% CM: <0.1), and this pattern parallelled the inhibitory effect on BN formation (Fig. 1). However, the level of OPN mRNA was not significantly changed by PC-3 CM (0% CM: 1.0; 5% CM: 0.9; 10% CM: 0.9; 20% CM: 1.0; 30% CM: 0.9).

## DISCUSSION

This study has shown that CM collected from PC-3 cell cultures decreases BN formation, ALPase activity, the mRNA level of the enzyme, and OCN in RC or ROS 17/2.8 cells. An interesting finding was that ALPase activity was inhibited by PC-3 CM but not by CM derived from Hela, HSG, and HSY cells, which were similar carcinoma cells used as controls. These results indicate that CM of the PC-3 cell line derived from prostatic carcinoma specifically inhibits the differentiation of these two osteoblastic cell lines. Human prostatic carcinoma is known to commonly metastasize to bone tissue by lymphatic and hematogenous routes and induce new bone formation [Chiarodo, 1991]. Some studies have shown that PC-3 CM increases [$^3$H]thymidine incorporation or proliferation of osteoblastic cells including UMR 106, SAOS-2,

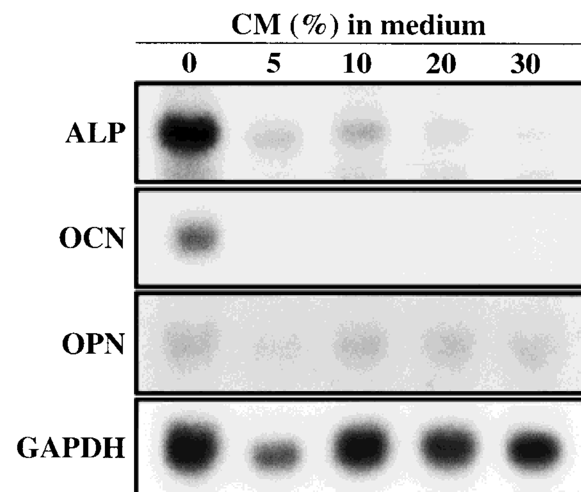


**Fig. 4.** Effect of PC-3 CM on ALPase, OCN, and OPN mRNA levels in ROS 17/2.8 cells. ROS 17/2.8 cells ($58.8 \times 10^4$) were inoculated in 60 mm dishes and cultured in medium containing 0–30% PC-3 CM for 6 days. The levels of ALPase, OCN, and OPN mRNA relative to that of GAPDH mRNA were analyzed by Northern blot hybridization. Similar results were obtained from two other separate experiments.

and human osteoblast cells [Simpson et al., 1985; Rabbani et al., 1990; Perkel et al., 1990], suggesting that a component in PC-3 CM might play a role in the osteoblastic metastasis of prostatic carcinoma. Prostatic carcinoma cells metastasize through bone formation and resorption. Therefore, we speculate that bone resorption may be necessary in order to create space for the growth of osteoblastic metastasis from prostatic carcinoma and that both inhibition of osteoblastic cell differentiation and activation of osteoclasts are important for this process. Martínez et al. [1996] showed that the concentrated PC-3 CM (at 20 μg/ml medium) decreased ALPase activity to 30% of the control level and intracellular calcium accumulation to one-seventh of the control level in RC cells. Our data also indicated that PC-3 CM inhibited the enzyme activity to a similar degree in both undifferentiated RC cells (24% of control) (Fig. 2A) and well-differentiated ROS 17/2.8 cells (36% of control) (Fig. 2C) and that, in parallel with the enzyme activity, BN formation by RC cells was decreased by 20% CM, which contained 1.2 μg protein (Fig. 1).

OCN is a noncollagenous $Ca^{2+}$ binding protein synthesized by mature osteoblasts including ROS 17/2.8 cells, and its production is regulated by certain calcitropic factors such as parathyroid hormone, 1,25-dihydroxyvitamin $D_3$ and prostaglandin $E_2$ [Noda et al., 1988; Price and Baukol, 1980; Lian et al., 1985]. It is known that OCN is a specific marker of osteoblastic activity and plays a significant role in mineralization of the bone matrix [Hauschka et al., 1989; Nishimoto and Price, 1980]. This protein is also thought to be closely related to bone metabolism at sites of prostatic carcinoma metastasis, since the serum content of OCN is significantly elevated in patients with such metastasis [Arai et al., 1992]. The present study demonstrated that PC-3 CM strongly blocked the expression of OCN mRNA (less than 0.1, compared to 1.0 in the control) in ROS 17/2.8 cells at a concentration of more than 5% (Fig. 4), supporting the possibility that PC-3 CM might have a catabolic action on bone metabolism in metastatic lesions. On the other hand, OPN, a phosphorylated glycoprotein synthesized mainly by osteoblasts and osteoclasts and deposited in the bone matrix, is thought to play an important role in bone remodeling [Nagata et al., 1991; Sodek et al., 1992; Heinegård et al., 1995]. In our study, PC-3 CM did not have a significant effect on OPN mRNA expression in ROS 17/2.8 cells (Fig. 4). It is reported that strong expression of OPN is evident immunohistochemically in breast cancer [Bellahcene and Castronovo, 1995] and that the level of OPN mRNA increases in many carcinomas, including those of the prostate, breast, lung, colon, and ovary [Brown et al., 1994]. However, the function of OPN and its regulatory role in bone metabolism in metastases from prostatic carcinoma are obscure, and these aspects will need to be studied in the future.

Kimura et al. [1992] showed that PC-3 CM increased the calcified area in cultures of the human osteoblastic cell line Tak-10. Extracts of prostatic carcinoma are known to increase ALPase activity in UMR 108 cells [Koutsilieris et al., 1987]. Moreover, Simpson et al. [1985] demonstrated that PC-3 CM had a bifunctional effect—increasing and decreasing the ALPase activity of UMR-106 cells at dilutions of 1:8 and 1:4 or above, respectively. At present, the mechanism of PC-3 CM action on osteoblastic cell differentiation remains unclear, but we speculate that PC-3 CM may contain an inhibitor as well as a stimulator of differentiation. It has been reported that various growth factors including TGF-β [Ikeda et al., 1987; Wilding et al., 1989], FGF [Story et al., 1987; Hierowski et al., 1987], EGF [Jacobs et al., 1988], and urokinase [Rabbani et al., 1990] are produced by PC-3 cells and secreted into their culture medium. These factors play significant roles in normal bone metabolism by regulating osteoblastic cell differentiation [Breen et al., 1994; Hock et al., 1990; Rodan et al., 1989; Antosz et al., 1989; Hoekman et al., 1991].

It has been reported that TGF-β, a multifunctional regulator of cell proliferation and differentiation, suppresses BN formation, ALPase activity, and OCN synthesis in RC cells [Antosz et al., 1989; Canalis and Lian, 1988] and inhibits OCN mRNA expression but stimulates ALPase activity in ROS 17/2.8 cells [Noda, 1989; Noda and Rodan, 1987; Pfeilschifter et al., 1987]. FGF and EGF also inhibit these osteoblastic markers in RC and ROS 17/2.8 cells but stimulate osteoblastic cell proliferation [Nicolas et al., 1990; Rodan et al., 1989; Antosz et al., 1989]. Based on the present results, which indicate the inhibition of BN formation, ALPase activity, and OCN mRNA expression by PC-3 CM without affecting the proliferation of RC and ROS 17/2.8 cells, the effects of CM differ

from those of TGF-β, FGF, and EGF. Moreover, we found that the fraction with a molecular mass of 50–55 kDa inhibited the ALPase activity of ROS 17/2.8 cells (data not shown). Because the molecular mass of this fraction is considerably different from those of TGF-β (23–25 kDa), bFGF (16 kDa), and EGF (6,045), the component in PC-3 CM responsible for the inhibitory action seems to be a molecule other than TGF-β, FGF, and EGF.

The level of urokinase-type plasminogen activator in prostatic carcinoma tissue is known to be higher than that in hyperplasia [Kirchheimer et al., 1984]. Rabbani et al. [1990] indicated that a $NH_2$-terminal fragment of urokinase was contained in PC-3 CM and that the high molecular weight (HMW) urokinase containing this peptide has mitogenic activity for SAOS-2 and RC cells. Because the molecular mass of HMW urokinase is about 55 kDa, similar to that of our fraction showing inhibitory action, we investigated the effect of HMW urokinase on ALPase activity in RC cells. HMW urokinase at 100 nM, which showed clear mitogenic activity in the study by Rabbani et al. [1990], inhibited ALPase activity by only 47% (data not shown). However, our PC-3 CM produced 63–77% inhibition, exceeding that of urokinase, at a concentration of 20% without any mitogenic activity, suggesting that the inhibitory factor present in CM is a component different from HMW urokinase.

In this study, PC-3 CM clearly inhibited the osteoblastic markers in RC and ROS 17/2.8 cells but did not affect osteoblastic cell proliferation. Previous reports showed that PC-3 CM stimulated ALPase activity, cell number, and DNA synthesis in several osteoblastic cells [Simpson et al., 1985; Rabbani et al., 1990; Perkel et al., 1990]. Although it is difficult to explain the contrasting actions of PC-3 CM on osteoblastic cell differentiation and proliferation, one possibility is that this CM may induce a different response in different types of osteoblastic cells [Noda and Rodan, 1987; Harris et al., 1994]. Alternatively, differences in the CM concentration used may be responsible. The present PC-3 CM did not cause a significant reduction of cell proliferation, showing values 92% and 82% of the control at concentrations of 20% (1.2 μg protein) and 30% (1.8 μg protein), respectively, and had no effect on the viability of ROS 17/2.8 cells (Fig. 3C). Kimura et al. [1992] also showed that 25% PC-3 CM induced little increase of DNA synthesis in Tak-10 cells (95% of control). In contrast, a 1:2 dilution of CM in culture medium and 10–50 μg CM protein/ml, higher than our CM concentration, stimulated osteoblastic cell proliferation [Simpson et al., 1984; Perkel et al., 1990]. These results suggest that CM does not have mitogenic activity at lower concentrations.

Our preliminary experiments have shown that the inhibitory activity of PC-3 CM on ALPase activity disappeared upon pretreatment with 200 μg/ml proteinase K and heating for 60 min at 100°C (data not shown). Therefore, the factors exerting an inhibitory effect on osteoblastic cell differentiation are thought to be protein-like. Although we have not clearly identified the factor present in PC-3 CM, the effect seems to be due to the action of a novel factor or the combined actions of several known factors. The inhibitory effect of PC-3 CM on osteoblastic cell differentiation may be involved in bone resorption in metastatic lesions of prostatic carcinoma, and the factor responsible is thought to have a very important role in metastasis at the cellular level. In future studies, we intend to isolate and identify this factor and clarify its relationship with osteoblastic metastasis of human prostatic carcinoma.

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