Prostate-Derived Soluble Factors Block Osteoblast Differentiation in Culture

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Abstract
Bone metastasis is a common event and a major cause of morbidity in prostate cancer patients. After colonization of bone, prostate cells induce an osteoblastic reaction which is not associated with marrow fibrosis (i.e., osteoblast but not fibroblast proliferation). In the present study we test the hypothesis that the tumoral prostatic cell line (PC-3) secretes factors that block the osteoblast differentiation process, resulting in an increase of the relative size of the proliferative cell pool. Our results, using fetal rat calvaria cells in culture, show that conditioned medium from PC-3 cells (PC-3 CM) stimulates osteoblast proliferation and inhibits both alkaline phosphatase (AP) activity (an early differentiation marker) and the mineralization process, measured as calcium accumulation (late differentiation marker). The inhibition of the expression of AP and mineralization depends on the presence of PC-3 CM during the proliferative phase of culture and suggests that both processes occur in a nonsimultaneous fashion. The inhibitory effect of PC-3 CM was not reverted by dexamethasone, which would indicate that prostatic-derived factors and the glucocorticoid do not share a common site of action. Measurement of the proliferative capacity of subcultures from control and treated cells demonstrates that PC-3 CM treatment induces the maintenance of the proliferative potential that characterizes undifferentiated precursor cells.

Key words: osteoblasts, calvaria, invasion, prostate, PC-3 cells, differentiation, metastasis

One of the characteristic features of advanced prostate cancer is its propensity to invade bone [1]. Growth of prostatic cancer cells into the skeletal tissue has been associated with high mortality rates and represents a leading cause of cancer-related deaths among males [2]. In vivo, prostate cells cause a sclerotic reaction in the infiltrated bone and impairment of the mineralization process [3,4]. In vitro, prostate-derived factors induce an osteoblastic proliferative reaction that leads to an increased number of osteoblasts in culture after the stimuli and an increase in thymidine incorporation into DNA [5].

Fetal rat calvaria cultures constitute an useful tool to study not only growth and differentiation of bone cells in vitro but also to evaluate the effect of foreign effectors on this process [6]. Under culture conditions, osteoblast calvaria cells are able to set the whole temporal sequence of events occurring during osteoblast differentiation in vivo: proliferation, establishment and maturation of extracellular matrix (ECM), and mineralization [7].

Studies performed using this experimental system have shown that conditioned media (CM) of a human prostate invasive cancer cell (PC-3) [8] stimulate thymidine incorporation in osteoblasts from rat calvaria cells [9]. These authors identified the amino terminal end of urokinase-like plasminogen activator (u-PA) as the component of PC-3 CM that exerts this mitogenic stimuli. However, there is no evidence whether this mitogenic effect of prostate-derived factors have some consequence on the expression of the differentiation pattern of osteoblasts in culture.

In the present study, we investigate the effect of media conditioned by a tumoral metastatic prostate cells (PC-3) on the differentiation of rat osteoblasts in culture. Our hypothesis is that the increased proliferation of osteoblasts in culture in response to prostatic factors could be consequence of a blockade of the differentiation process or by a specific mitogenic stimuli. The cells would therefore maintain an undifferentiated condition, avoiding their progress into differentiation pathways which lead to the expression of the mineralized phenotype.
MATERIALS AND METHODS

Cells

PC-3 cells correspond to a human prostate tumoral cell line derived from a bone metastasis [8]. These cells grow in DMEM-F-12 culture media enriched with 10% fetal calf serum (FCS) and were purchased from American Type Culture Collection (ATCC) (Maryland).

In order to prepare conditioned media (CM) from these cells, confluent cultures were incubated for 48 h in a serum-free medium. Once collected, cell-free CM was concentrated with polyethylene glycol (MW 8,000) and dialyzed against phosphate buffer saline (PBS).

Primary cultures of osteoblast-like cells from rat calvaria were prepared essentially as indicated by Owen [7]. Briefly, calvaria from fetal Wistar rats of 21 days of gestation were isolated and incubated at 37°C in PBS for 20 min. After this incubation, bones were subjected to sequential digestion of 20, 40, and 90 min in a mixture of 0.02% collagenase type II (Worthington, NJ) and 0.25% trypsin (Sigma, St. Louis, MO). Cells of the first two digests were discarded, and those released from the third digestion were washed and plated in minimal essential medium (DMEM) (Sigma) supplemented with 10% fetal calf serum (FCS) (Gibco BRL, Gaithersburg, MD) in 16 mm plates (Nunc, Roskilde, Denmark) at a density of $5 \times 10^4$ cells per well. At confluency (day 6), control cultures were fed with mineralization media (MM) consisting of BGJb medium (Sigma) supplemented with 10% FCS and 50 μg/ml of ascorbic acid and 10 mM β-glycerol phosphate. Cells received this MM throughout all the experimental period. In experiments where cultures were treated with conditioned media from prostate cells, the media was added from day 3 of culture at a final concentration of 20 μg/ml. In experiments where dexamethasone was used, hormone was added at concentrations of $10^{-7}$ and $10^{-6}$ M on each feeding day.

Proliferation Assays

Proliferative potential of rat osteoblast-like cells was assayed by two methods: 1) measuring the increase in cell number during the proliferative period of the culture and 2) $^3$H-thymidine incorporation into DNA. The rate of DNA synthesis at each time indicated in the figures was measured by incorporation of $^3$H-thymidine (67 Ci/mmole; ICN Radiochemicals, Irvine, CA). In these experiments, cells growing in the presence or absence of prostate-derived CM were incubated with 2 mCi/ml of $^3$H-thymidine for 4 h. At the end of this period, the cellular monolayer was rinsed three times with PBS and incubated two times with 5% (w/v) trichloroacetic acid (TCA) for 5 min. Cells were solubilized in 0.5 ml 10% SDS and counted in a scintillation counter.

Some experiments where designed in order to measured true proliferative potential, avoiding the intrinsical inhibition of confluency. Cells derived from control and PC-3 CM-treated cultures were harvested by trypsinization at different times of culture of the primary culture. Then cells were plated in the absence of conditioned media for 24 h. During the last 4 h of culture, cells were pulsed with $^3$H-thymidine according to the procedure described previously.

Alkaline Phosphatase Determination

At days 5, 8, 12, 15, 18, 21, 25, and 28 of culture, cells were assayed for alkaline phosphatase (AP). Cells were scraped and stored at -80°C together with culture medium containing 0.2% Triton X-100 in order to avoid loss of soluble activity. After thawing, the cell suspension was sonicated (23 kHz), and the cellular debris was separated by centrifugation (13,000g, room temperature). Enzymatic activity was assayed in the supernatant of this suspension in a total volume of 2.0 ml in 50 mM buffer carbonate/sodium bicarbonate, pH 9.6, containing 1 mM MgCl$_2$ and 1 mM Sigma 104 substrate. After 20 min, the reaction was stopped with 0.5 ml of 3 N NaOH, and color developed was measured at 405 nm.

Calcium Determination

Cells were washed three times with calcium-free PBS prepared with double deionized water, scraped, and resuspended in a solution of 0.5 N HCl. Cells were sonicated, and the acid supernatant was used for calcium determination in an Atomic Absorbtion Spectrophotometer (Perkin Elmer model 2280).

RESULTS

In order to evaluate the effects of conditioned media from PC-3 cells (PC-3 CM) on osteoblast proliferation, two types of experiments were performed. Figure 1 shows the increase in number of osteoblast-like cells cultured in the presence or absence of PC-3 CM. As Figure 1 shows, at
day 11, cells cultured in the absence of CM start to reduce their proliferation rate; at the same time, cells cultured in the presence of CM are still under linear proliferative growth. Cells incubated with PC-3 CM have higher DNA synthesis than controls at any time during the proliferative phase of the culture (Fig. 1, insert). The apparent discrepancy between cell number and \(^{3}H\)-thymidine incorporation from day 7 can be explained by the fact that our cultures start to reach confluency at day 6. Therefore, there is in fact agreement between thymidine incorporation levels and the decrease in exponential rate of increase in cell number shown in Figure 1.

Alkaline phosphatase (AP) activity of calvarial osteoblasts was evaluated during the entire differentiation period in control cells and cultures enriched with PC-3 CM. As Figure 2 shows, in control cultures AP activity follows a classical time course, with a maximum at day 15. Osteoblast-like cells cultured in the presence of PC-3 CM display an enzymatic pattern similar to that in control cultures but with a maximum value equivalent to 30% of the control level.

With the purpose of further characterizing the temporal events by which PC-3 CM inhibits AP activity, osteoblast cultures were subjected to different times of exposure to PC-3 CM. Data in Figure 3 show that a 24 h incubation (days 1–2) with PC-3 CM does not inhibit the normal expression of osteoblastic AP during 4 weeks of culture (Fig. 3A). Prolongation of the time of exposure to PC-3 CM from days 1–5 (Fig. 3B) results in a 50% decrease in AP activity at day 15. Incubation of rat osteoblast with PC-3 CM from days 5–11 inhibits the temporal expression of AP activity to the same extent as the level present in cells cultured with PC-3 CM by the entire period of culture. Cells cultured from day 11 to 25 express AP activity as control cells that were cultured in the absence of any exogenous agent (Fig. 3D), showing no inhibition of AP activity in response to culturing with PC-3 CM during this period.

Fig. 1. Proliferation of osteoblasts. Control (filled symbols) and PC-3–CM treated cells (hatched symbols) were cultured for different periods, and cell number was scored by hemocytometer. DNA synthesis was assessed by \(^{3}H\)-thymidine incorporation (insert). Each point represent mean ± S.E.M. of three determinations.
Effect of PC-3 Medium on Osteoblasts

Mineralization, estimated by calcium accumulation into the cell layer, was measured during the entire culture period. As Figure 4 shows, control cells sharply increase their calcium content from day 12. In contrast, PC-3 CM–treated cells show only a marginal calcium accumulation, reaching final values seven times lower than their control counterparts.

To investigate if PC-3 CM inhibits mineralization through a sequence of temporal events similar to those observed in AP activity, experiments were set up using different times of exposure to PC-3 CM. Data in Figure 5 show that incubation with prostate-derived factors during the first 5 days or during the last 12 days does not produce alterations in the mineralization pattern of rat osteoblasts (Fig. 5A,D). When the cells were incubated in the presence of PC-3 CM between days 5 and 11, a 50% inhibition of calcium accumulation was observed (Fig. 5B). Only when stimuli were present between days 5 and 15 was an inhibition rate similar to that reached in the permanent presence of PC-3 CM observed (Fig. 5C).

In order to gain a better insight with respect to the mechanism of action of PC-3 CM on the osteoblastic pattern of differentiation, we measured AP activity in experiments of competition with dexamethasone (Dex), a well-known inducer of osteogenesis in vitro [10]. Rat calvaria osteoblasts were cultured for 14 days under the described conditions and from day 4 with the addition of Dex (10⁻⁷ and 10⁻⁸ M) or 20 μg of PC-3 CM or a mixture of both compounds. As Figure 6 shows, Dex was not able to revert the inhibition of prostate-derived factor at both hormone concentrations studied.

To demonstrate that PC-3 CM–treated cells do not express differentiation markers because of a blockade of the differentiation process, we measured true proliferative potential after releasing the cells from the intrinsic inhibition of confluence. For this, we measured the proliferative potential of control and PC-3 CM–treated cells after trypsinization. Osteoblast-like cells from primary culture on days 5, 8, and 10 were subcultured, and DNA synthesis was evaluated in these secondary cultures. As shown in Figure 7, ³H-thymidine incorporation by subcultures from control cells decays in a linear fashion with increasing time in primary culture. However, PC-3 CM–treated cells retain some proliferative potential and incorporate more ³H-thymidine than control cells. This is particularly noticeable at day 10, when control cells begin to differentiate (in terms of AP expression) and lose their proliferative potential, while PC-3 CM–treated cells maintain a similar capacity to incorporate thymidine.

**DISCUSSION**

The experiments presented in this study were conducted to investigate the proliferative response of cultured osteoblasts to conditioned media from tumoral prostatic cells and the expression of the differentiation program of osteoblast-like cells.

PC-3 cells—a cell line that secretes soluble factors which specifically stimulate proliferation of osteoblast-like cells—has been used as a model for the interaction between invasive prostatic cells and bone [11]. It has been suggested that PC-3 CM stimulates osteoblast proliferation by a mechanism which is different to that of known bone mitogens such as TGFβ, IGF-I, or IGF-II [12].

Growth and differentiation of osteoblasts have been studied using cultures of fetal rat calvaria. With this model, a reciprocal and functionally coupled relationship between proliferation and differentiation has been proposed by Stein and Lian [13]. These authors define a three-step temporal pattern, which begins with the amplification of a proliferative pool—period in which cells maintain an undifferentiated phenotype—followed by a period in which cells produce an
extracellular matrix and where AP is expressed maximally and ending with a period during which mineralization occurs [14].

A well-documented hypothesis derived from this model sustains the existence of two transition points (in which genes are selectively activated or suppressed) during the progressive acquisition of the bone cell phenotype. The first point is associated with the completion of proliferation when genes for cell cycle and cellular growth control are downregulated and expression of genes encoding protein for ECM maturation and organization is initiated. The second point is at the onset of the ECM mineralization [7].

Results from our study verify the capacity of PC-3 CM to stimulate replication in osteoblast-like cells, previously reported by Rabani et al. [9]. Measuring either the increase of cell number or $^3$H-thymidine incorporation into DNA, the enrichment of culture media with 20 μg of PC-3 CM induces a mitogenic response that is more evident close to the time of confluency.
Effect of PC-3 Medium on Osteoblasts

The cited study attributes to u-PA present in PC-3 CM the capacity to stimulate cell replication. In order to compare the experimental conditions in which our results were obtained and those utilized by Rabani et al. [9], we measured the relative activity of u-PA present in PC-3 CM and evaluated the mitogenic stimuli of commercially purified high molecular weight u-PA. In our hands, the effective activity of u-PA in 20 μg/ml of PC-3 CM is equivalent to one-fourth of the u-PA activity necessary to produce the same osteoblastic mitogenic response as PC-3 CM (data not shown). This result suggests that components in PC-3 CM, other than u-PA, are playing a role in this proliferative stimuli.

The time course of AP activity shows that cultured osteoblasts incubated in the presence of PC-3 CM express only a minor proportion of the enzyme present in control cells. This would suggest a PC-3 CM–mediated specific inhibition of the enzyme. Such inhibition could be present in all cells, assuming a homogeneous degree of differentiation, or in a specific cell population sensitive to changes in the AP expressing phenotype, assuming a heterogeneous degree of differentiation.

Incubation of bone cells with PC-3 CM for 24 h or during the second half of the culture period did not change the expression pattern of AP (Fig. 3A,D). However, exposure between days 4 and 12 produces the same results as a total exposure time of the maximum inhibition pattern (Fig. 3C). These results indicate that PC-3 CM affects AP expression only if it is present during the proliferative phase. It remains to be elucidated whether this event coincides with an inhibition of AP gene expression.

PC-3 CM also inhibits osteoblast mineralization. Calvaria cells cultured in the presence of

Fig. 5. Time course of mineralization of osteoblast after incubation for different periods of time with PC-3 CM. Mineralization, evaluated as calcium accumulation in the cell layer, was measured after incubating cells under the following conditions: control cells (filled circles), cells treated with PC-3 CM during the entire period of culture (filled squares), and cells treated with PC-3 CM only during fixed periods along the assay (open triangles). The length of these periods is indicated by a horizontal bar in each figure. The figure shows a representative result from three separate experiments.
Fig. 6. Effect of PC-3 CM and Dex on the activity of AP at day 14 of culture. AP activity, measured as indicated in Materials and Methods, was evaluated for the following conditions: 1: Control. 2: Cells treated with 20 μg/ml of PC-3 CM. 3: Cells treated with 10⁻⁷ Dex. 4: Cells treated with 10⁻⁸ Dex. 5: Cells treated with 10⁻⁷ Dex and 20 μg/ml PC-3 CM. 6: Cells treated with 10⁻⁶ Dex and 20 μg/ml PC-3 CM. Each point represents the mean ± S.E.M. of three determinations of two separated experiments.

PC-3 CM do not accumulate calcium as control cells. The inhibition of the mineralization process using selected times of incubation with PC-3 CM results in a pattern similar to that observed in AP activity. However, these similarities result from different times of exposure. For instance, 50% inhibition of AP activity is reached when cells are in the presence of PC-3 CM during the first 5 days of culture. In this condition, mineralization is not affected (Figs. 3B, 5A). Total inhibition of AP activity is obtained with an exposure time between days 4 and 11. Under this stimuli, mineralization decreases by 50% (Figs. 3C, 5B). To obtain an inhibition of mineralization with a pattern similar to that in the total exposure period, we need to maintain the PC-3 CM until day 15 (Fig. 5C).

The above mentioned results suggest that the inhibition of the expression of AP and mineralization does not occur in a simultaneous fashion. This fact can be explained by the presence of coordinated activity of two or more sets of genes which regulate the acquisition of mature phenotype, whose activation occurs at the transition points [13]. Similar findings have been obtained by in situ hybridization experiments in rat fetal calvarial-derived osteoblasts, in which a distinct temporal response has been described in response to additions of 1,25 (OH)₂D₃ and Dex. In such studies, there was a narrow window of response, indicating that cell responsiveness depends on the specific stage of differentiation [15].

The biochemical events that characterize osteoblast growth and differentiation are susceptible to modification by a variety of agents [16]. In vitro, glucocorticoids induce cells of the osteoblastic lineage to differentiate into mature cells expressing the osteoblastic phenotype [17]. In our experiments, the stimulatory effects of Dex on AP activity (60% stimulation above control) was not reverted by the presence of prostatic factor. Thus, although Dex and PC-3 CM exert their action during the proliferative phase [18], they appear to have separate and different mechanisms of action at the molecular level.

With the purpose of confirming whether PC-3 CM blocks the differentiation process, we estimated in secondary cultures the loss of proliferative potential in cells with different degrees of differentiation due to different periods of culture. Cells derived from control cultures lose their proliferative potential in subculture in a linear fashion that depends on the time in primary culture. In PC-3 CM–enriched cultures, the cells retain some proliferative capacity in subculture, and this trait is more evident in cells that are near the onset of the differentiation period. In light of these results we suggest that PC-3 CM produces a blockade on the progress of these cells through the differentiation process.
These cells maintain traits of the nondifferentiated phenotype such as a high proliferative potential. Similar results have been obtained with the addition of TGFβ in cultures of fetal rat calvaria cells [19,20]. In this case, however, stimuli on cell proliferation occur in two phases: at low cellular densities where TGFβ inhibits proliferation and at higher cell densities where the effect is clearly stimulatory. Nevertheless, the most important action of this growth factor is to prevent the programmed cellular differentiation into a mature osteoblast [20].

Our data suggests that cells incubated in the presence of PC-3 CM retain some of the characteristic traits of the undifferentiated precursor (i.e., low levels of AP expression and unmineralized phenotype). These results could be interpreted at least in two different ways:

1. The increased proliferative capacity of rat calvaria osteoblast after PC-3 CM incubation is a reflection of a recruitment of a pool of proliferative precursors as result of a blockade in the differentiation process.
2. PC-3 CM represents a specific stimuli for cell replication. In consequence, cells would be maintained in a permanent proliferative status which inhibits activation of differentiation genes.

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