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PRINCIPAL INVESTIGATOR: Nora M. Navone, Ph.D.

CONTRACTING ORGANIZATION: University of Texas
M.D. Anderson Cancer Center
Houston, Texas 77030

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FOREWORD

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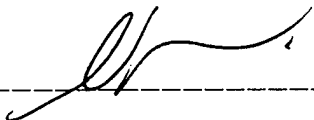
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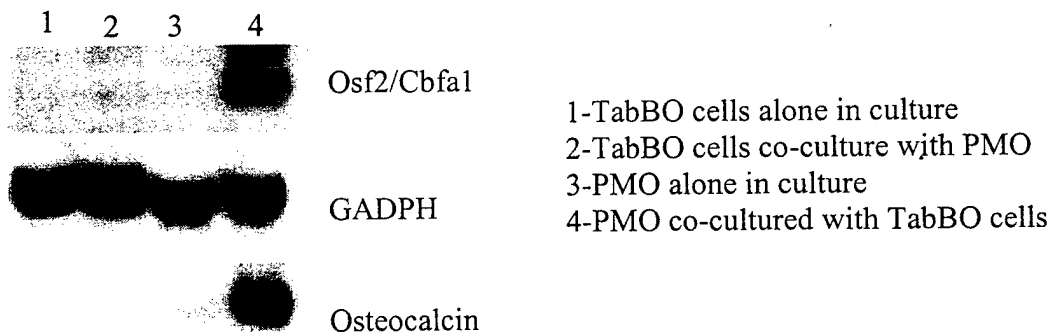
INTRODUCTION

Clinical prostate cancer is dominated by complications arising from bone metastases (BM). BM of prostate cancer are characteristically blastic, is the first site of androgen-independent progression, and account for a large portion of the clinical morbidity. The importance of BM in prostate cancer is reflected in the remarkable tendency of prostate cancers to develop BM as the only site of progression. This strongly suggests that the interaction of malignant epithelial cells with the bone microenvironment actively contribute to the lethal progression of prostate cancer. Consequently, understanding the factors that determine or influence the tropism of prostate cancer cells to bone may lead to treatments that prevent or reverse prostate cancer metastases. We have developed an *in vitro* model system that reflects the most common molecular and cellular features of prostate cancer BM. The model consists of prostate cancer cell lines MDA PCa 2a or MDA PCa 2b (the TabBO cells) co-cultured with primary mouse osteoblasts (PMO). The two cell types share medium but are not in physical contact because the prostate cancer cells are plated in cell-culture inserts. This co-culture increase proliferation of both the PMO and the TabBO cells, a proliferate response not observed with HeLa cells or breast cancer cell lines. This in turn indicates that the interaction is not universal and may be specific for prostate cancer cells and osteoblasts. This *in vitro* model system thus exhibits one of the hallmarks of prostate cancer BM and therefore can be used for studying, at the molecular level, the interaction of prostate cancer cells and osteoblasts.

BODY

Task 1. To determine whether prostate cancer cell lines alter the expression of osteoblast-specific genes such as *Osf2/Cbfa1* and thereby alter osteoblast differentiation.

Regulation of normal bone formation involves sequential expression of growth factors and transcription factors by osteoblasts as they proliferate and ultimately differentiate. The osteoblast, the bone-forming cell, is a cell of mesenchymal origin that when terminally differentiated produces most of the protein present in the bone extracellular matrix (ECM) and controls the mineralization of this ECM. *Osf2/Cbfa1* is an osteoblast-specific transcription factor, a regulator of osteoblast differentiation, and the earliest and most specific marker of osteoblast differentiation known (1). In our model system, we first studied the expression of *Osf2/Cbfa1* and osteocalcin, the only gene expressed in osteoblasts and in no other ECM-producing cells (2). Northern blot analysis of PMO co-cultured with TabBO cells versus PMO cultured alone revealed increased expression of *Osf2/Cbfa1* and osteocalcin in osteoblasts after 4 days in co-culture. Similar results were obtained in three separate experiments.



However, we could not reproduce these results in subsequent experiments or reproduce the proliferative response observed in the initial experiments. For that reason, we concluded that our model system requires further work to establish optimal conditions that would make the system reproducible. We decided to determine the optimal cell density that each compartment should have at the beginning of the experiment so that a consistent effect in terms of cell number would be observed after 4 days in co-culture. The initial cell density was chosen so that we would have approximately 70% confluence of in all control culture at the end of the experiments, therefore allowing an effect on cell number to be assessed in the co-cultures. To establish optimal cell density we focused on one compartment at a time. After a series of experiments using different densities of PMO in co-culture with TabBO cells, we found that using 5,000 PMO cells/cm² produced reproducible results in three subsequent experiments. Therefore, we used 5,000 PMO cells/cm² as the optimal cell density in all subsequent experiments

To test the optimal cell density of the malignant epithelial cell compartment in our model system, we performed a series of experiments with different densities of TabBO cells (100,000; 150,000; and 200,000 cells/cm²), and HeLa cells (1,000; 2000; and 3,000 cells/cm²). We found the optimal cell density to be 100,000/cm² for TabBO cells and 8,000/cm² for HeLa cells.

The TabBO lines (MDA PCa 2a and MDA PCa 2b), which we recently established in my laboratory are the first cell lines derived from a bone metastasis of prostate cancer that exhibit key features of prostate cancer: prostate-specific antigen (PSA) expression and androgen responsiveness (3). To better characterize our model system, we decided to include other prostate cancer cell lines as controls, namely PC3 (4) and LNCaP (5). PC3 is a bone-derived, highly undifferentiated (androgen-unresponsive and non-PSA-expressing line) that produces osteolytic bone metastases in experimental systems *in vivo*. The LNCaP line is the only other human prostate cancer cell line available that is androgen responsive and expresses PSA. It is also only weakly tumorigenic in experimental systems and is derived from a lymph node metastasis of an androgen-independent prostate cancer. As before, we assessed the optimal cell density and found it to be 50,000 cells/cm² for LNCaP.

Using the optimal conditions established *a priori*, we then performed co-culture experiments using the different cell lines and subsequently assessed cell number after 4 days in co-culture (cell number in co-cultures was always compared with cell numbers in cultures growing alone). We first assessed cell number in the PMO compartment. As shown in Fig 1 co-culture with TabBO cells increased the proliferation of PMO. In contrast, co-cultures of PMO with LNCaP cells produced a smaller increase in PMO, and co-culture of PMO with HeLa cells had no effect in the number of PMO. These experiments were performed three times, and the proliferative response of PMO to TabBO cells was very reproducible. In contrast, the response of PMO to the LNCaP cells sometimes varied, whereas the proliferative response of PMO to HeLa cells ranged from none to a decrease in cell number. We are in the process of assessing the statistical significance of these results. However, given the magnitude and reproducibility of the proliferative response of PMO to TabBO cells, we are certain that the response is biologically meaningful.

In the same experiments, we also assessed the proliferative response of the malignant epithelial cell compartment after co-culture with PMO. The number of cells in the epithelial malignant compartment is given in Fig 1. It can be seen that TabBO cells and LNCaP cells showed a proliferative response after 4 days in co-culture with PMO. No proliferative response was observed in HeLa cells. The increased in cell number after 4 days in co-culture was more pronounced for the TabBO cells. However, because the magnitude of the proliferative response

of the TabBO cells varied in different experiments, subsequent experiments will be performed to further define the optimal growth conditions for the TabBO cells that will make the system more reproducible and reliable.

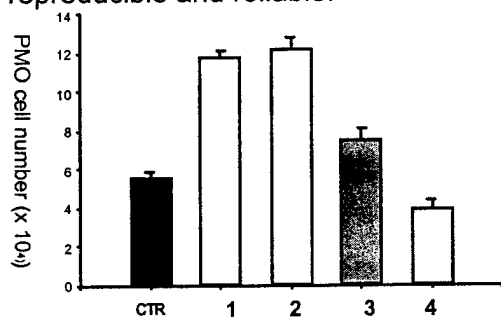


Fig 1. Cell number in PMO cultures grown alone for 4 days as a control (CTR) and in co-culture with MDA PCa 2b (1), MDA PCa 2a (2), LNCaP (3), and HeLa (4) cells grown for 4 days. The cells were harvested and counted with a hematocytometer. The control culture and each different co-culture were assayed in six times.

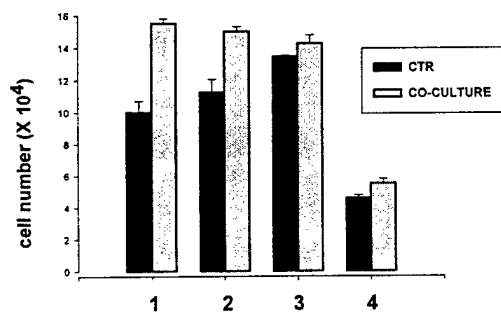


Fig 2. Cell number in cultures of MDA PCa 2b (1), MDA PCa 2a (2), LNCaP (3) and HeLa (4) cells grown alone as controls for 4 days (CTR) and after 4 days in co-culture with PMO. Cells were harvested and counted with a hematocytometer. The control cultures and each co-culture were assayed six times.

We also assessed the optimal cell number to be used in co-culturing PC3 and PMO. As Fig 3 illustrates, co-culture of PC3 cells with PMO had either no effect or caused a decrease in PMO number, depending on the initial number of PC3 cells used. These results agree with the fact that the PC3 cell line produces osteolytic metastases *in vivo* and support the notion that our model system reflects the interaction between prostate cancer cells and osteoblasts in prostate cancer BM. As illustrated in Fig 4 when we used 4,000 or 8,000 cells/cm², the PC3 cell number increased after 4 days in co-culture with PMO. These results were reproduced in two separate

experiments, and we have therefore we decided to use 8,000 cells/cm² in subsequent experiments. We will now perform an experiment using PC3 cells at the same time as the other cell lines to see whether the same results are obtained when all cells are co-cultured with the same batch of PMO at the same time.

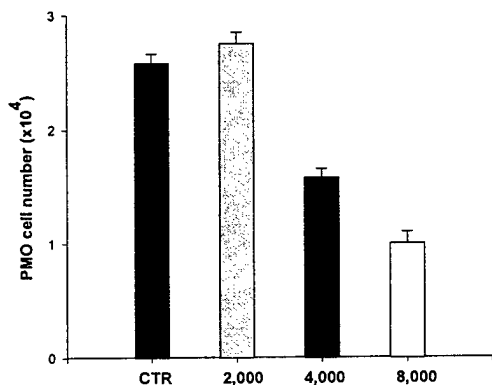


Fig 3. Cell number of PMO cultures growing alone for 4 days (CTR) and after 4 days in co-culture with different amounts of PC3 cells (2,000; 4,000; 8,000/cm²). The cells were harvested and counted with a hemacytometer. The control culture and each co-culture were assayed six times.

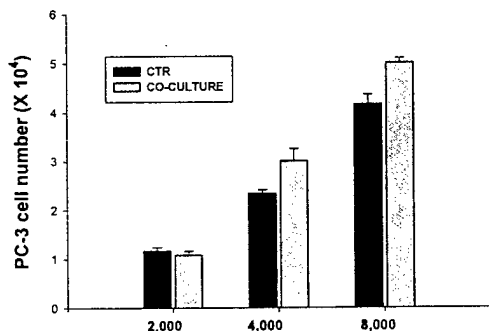


Fig 4. Cell number of PC3 cultures growing alone for 4 days (CTR) and after 4 days in co-culture with PMO. The cells were harvested and counted with a hemacytometer. The control culture and each co-culture were assayed six times.

Because the effect of cancer cells on PMO in our model system is very reproducible, we decided to further define this compartment. The development, differentiation, and maturation of the osteoblast phenotype in primary cultures of rodent calvarium osteoblasts are well characterized. In prolonged culture these cells undergo a defined series of events from

proliferation to maturation, and express osteopontin, alkaline phosphatase, bone sialoprotein, and osteocalcin and, ultimately, form mineral. Induction of the osteoblast phenotype by a variety of growth factors, including glucocorticoids and ascorbate, has been reported in murine and human bone cell cultures. Also, it has been observed that primary mouse osteoblasts require the presence of bone morphogenic protein 2 (BMP-2) to produce mineral formation in vitro (Dr Peleg, personal communication).

We have established a collaboration with Dr Sara Peleg (The University of Texas M.D. Anderson Cancer Center) to study the sequence of events that osteoblasts undergo when growing alone and when co-cultured for 4 days in our model system. For that purpose we are growing PMO in the presence and absence of TabBO cells for 4 days in α -modified minimum essential medium and 10% fetal bovine serum. After 4 days in co-culture, the two compartments are separated, and the PMO (from the co-cultures and the controls) are placed in differentiation medium (50 mg/ml ascorbic acid and 10 nM sodium beta-glycerophosphate) in the presence and absence of BMP-2. Then after 21 days, the cultures are tested for expression of osteocalcin and alkaline phosphatase and for matrix deposition (von Kossa staining) at different time points. Osteoblast cells comprise a great diversity of morphologies and activities, ranging from preosteoblasts to osteoblasts and osteocytes. These different morphotypes are generally considered different maturation stages of the same cell lineage originating from the proliferation and differentiation of osteoprogenitor cells of pleiomorphic or fibroblastic morphology. These studies should provide evidence that the PMO are composed of osteoblasts and osteoblast precursors that are able to differentiate and produce matrix deposition. Preliminary results from these experiments have shown that after 12 days in the presence of differentiation medium and BMP-2, PMO are able to produce matrix. Moreover, the matrix deposition is proportional to the number of cells present in the culture. These results suggest that TabBO cells induce proliferation of PMO that are able to differentiate into mature osteoblasts, which in turn produce mineralized matrix. These results are in agreement with our earlier findings of increased expression of *Osf2/Cbfa1* mRNA and osteocalcin mRNA in PMO after 4 days in co-culture. However, we have no explanation for our inability to reproduce those results.

Task 2. To characterize and purify the soluble factors that are present in the conditioned medium (CM) produced by prostate cancer cells and that are responsible for the alterations in the cell growth of osteoblasts.

Task 2 has been postponed until we feel that our model system is reproducible enough to produce meaningful results.

Task 3. To determine whether co-culturing osteoblasts with human prostate cancer cells affects the expression of hormone-responsive genes such as PSA and the expression of cell cycle-related genes in MDA PCa 2 cells.

To accomplish task 3 we examined p53 and bcl-2, because they are known to have a role in the progression of prostate cancer to the androgen-independent, metastatic phenotype (6-10); the p21^(WAF1/CIP1) (p21) gene because it is a downstream target of p53 (11); and the PSA gene because it is the best characterized downstream target of the androgen receptor (12) and is widely used as a marker of progression in prostate cancer (13). Northern blot analysis of PSA, p53, p21, and bcl-2 showed no difference in expression levels in cells grown alone versus those grown in co-culture (Fig 5). We then decided to study the expression of these genes at the protein level. In preliminary experiments we found a slight increase in expression of p21 in

TabBO cells after 4 days in co-culture but no changes in bcl-2 or p53 expression levels (Fig 6). We are now repeating these experiments to assess the reproducibility of our findings.

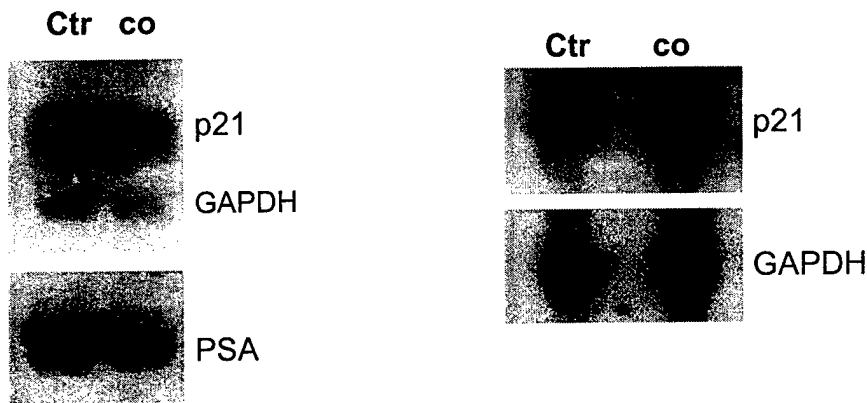


Fig 5- Northern blot analysis of p21, p53, and PSA expression in MDA PCa 2b cells grown alone (Ctr) and after 4 days in co-culture (co). GAPDH was used as a loading control.

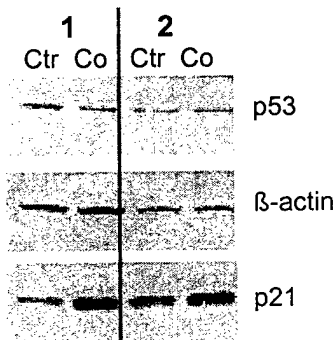


Fig 6. Western blot analysis of MDA PCa 2a (1) and MDA PCa 2b (2) cells grown alone (Ctr) and after 4 days in co-culture (Co) performed with monoclonal antibodies to human p53 and human p21. β -actin was used as a loading control.

Task 4. To characterize and purify soluble factors present in the conditioned medium (CM) produced by osteoblasts and that are responsible for the alteration in cell growth of prostate cancer cells.

Task 4 has been postponed until we feel that our model system is reproducible enough to produce meaningful results.

KEY RESEARCH ACCOMPLISHED

Found optimal conditions to study the interaction between prostate cancer cells and PMO in an *in vitro* model system that we established.

Using those conditions, we found that the well differentiated, bone derived prostate cancer cells (TabBO lines) induce a proliferative response in the PMO.

PMO that results from the proliferative response are able to differentiate into mature osteoblasts. This suggests that our model system may be suitable to study the molecular and cellular events involved in the new bone formation observed in prostate cancer bone metastases.

We have found that PC3 prostate cancer cells, a cell line that produces osteolytic bone metastases in experimental models, do not induce proliferation of PMO in our model system. This further suggest that our model system reflect the interaction that occur between prostate cancer cells and osteoblasts in prostate cancer bone metastases

REPORTABLE OUTCOMES

Abstract

-2000 AACR Annual Meeting in San Francisco-Poster presentation entitled: Osteoblast-Prostate Cancer Cells Interaction Attenuates the Antiproliferative effect of Vitamin D3. Navone, N.M., Yang, J., Olive, M., Logothetis, C.J., and Peleg S.

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Development Rational Basis of Bone-Homing Therapies for Prostate Cancer
Principal Investigator: Nora M. Navone, M.D., Ph.D.
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Total Award \$50,000

Presentation

1999 CaP CURE's Annual Scientific Retreat at The Hyatt Regency at Lake Tahoe, Nevada.
"Rational Basis for Bone-Homing Therapies for Human Prostate Cancer".

CONCLUSIONS

We have established the optimal conditions for growing prostate cancer cells in co-culture with PMO. Using those conditions, we defined the effect that prostate cancer cells have in PMO in our model system. This effect reflects the interaction between prostate cancer cells and osteoblasts in prostate cancer bone metastases. Therefore we conclude that our model system may be suitable to study the molecular and cellular events involved in the new bone formation observed in prostate cancer bone metastases.

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