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Three-dimensional cancer-bone metastasis model using *ex-vivo* co-cultures of live calvarial bones and cancer cells

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ABSTRACT

One of the major limitations of studying cancer-bone metastasis has been the lack of an appropriate *ex-vivo* model which can be used under defined conditions that simulates closely the *in vivo* live bone microenvironment in response to cancer-bone interactions. We have developed and utilized a three-dimensional (3D) cancer-bone metastasis model using free-floating live mouse calvarial bone organs in the presence of cancer cells in a roller tube system. In such co-cultures under hypoxia and a specifically defined bone remodeling stage, viz., resorption system, cancer cells showed a remarkable affinity and specificity for the "endosteal side" of the bone where they colonize and proliferate. This was concurrent with differentiation of resident stem/progenitor cells to osteoclasts and bone resorption. In contrast, under bone formation conditions this model revealed different pathophysiology where the breast cancer cells continued to induce osteoclastic bone resorption whereas prostate cancer cells led to osteoblastic bone formation. The current 3D model was used to demonstrate its application to studies involving chemical and biochemical perturbations in the absence and presence of cancer cells and cellular responses. We describe proof-of-principle with examples of the broad versatility and multi-faceted application of this model that adds another dimension to the ongoing studies in the cancer-bone metastasis arena.

1. Introduction

Bone metastases occur in a majority of patients with advanced breast and prostate cancers for reasons that are still not well understood. While bone is not the only organ where metastasis can occur, bone is one of the preferred sites [1]. Once in the blood-stream tumor cells reaching the bone marrow adhere to the "endosteal" surface and colonize bone. The site and formation of bone lesions is determined by multiple cellular and molecular interactions between cancer cells and the bone microenvironment [2–4]. These co-operative interactions rely on factors that are secreted by cancer cells that stimulate proliferation and differentiation of osteoblasts and osteoclasts with a net effect of either bone resorption or bone formation [5,6]. Bone undergoes remodeling continuously throughout life with the coupling of formation and resorption involving osteoblasts and osteoclasts, respectively. The physico-chemical properties of the bone microenvironment and its cellular components play a significant role in pathogenesis. To date a clear understanding of the cellular and molecular mechanisms involved in cancer-bone interactions and other factors that may play a critical role are limited. This is partly due to a lack of an ideal experimental model(s) that could recapitulate these complex multicellular physiological events under defined conditions to effectively study them at physical, cellular and molecular levels.

A variety of two-dimensional (2D) cell cultures and *in vivo* animal models have been developed and used to investigate the mechanistic complexities of cancer-bone metastasis [7–9]. It is well known that cell–cell and cell–matrix interactions play a major role in tumor morphogenesis and cancer metastasis. However, thus far there is no ideal model that incorporates all of these *in vivo* physiological events. In a 2D cell culture system there is no three-dimensional (3D) microenvironment consisting of an extracellular

Abbreviations: ECM, Extracellular matrix; PGE2, prostaglandin E2; 2D, two-dimensional; 3D, three-dimensional; PTH, parathyroid hormone; MMP-3, matrix metaloproteinase 3; NR, neutral red; Cox2, cyclo-oxygenase-2; ELISA, enzyme-linked immunosobent assay; DMEM, Dulbecco's Modified Eagle's Medium; EDTA, ethylene diamine tetraacetic acid; IL-6, interleukin-6.

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matrix (ECM) comparable to native tissue. This lack of cell–matrix interaction has an adverse impact by deviating from the *in vivo* condition which results in changes in cancer cell phenotype and gene expressions [10,11]. Such inconsistencies may provide less reliable data with limitations for translating to clinical applications. While *in vivo* animal models have more relevance and lack the disadvantages of the 2D cell culture systems, they too have limitations. There is the ethical and cost issues which can restrict the number of experiments to obtain statistically reliable data. The pathology and disease progression may not be accurately represented between humans and animal models. In response to demands for new and improved cancer-bone metastasis models, more recent studies have attempted to overcome these limitations by the development of three-dimensional (3D) *in vitro* models. These include the use of cancer cells embedded in collagen type I gel and co-cultured with mouse calvarial bone directly in contact with the gel or separated by a filter in a normal culture dish and conditions [12]. A variation of such co-cultures was the development of cancer cells grown on the bottom of the culture dish with calvarial bone placed on an elevated metal grid under normal culture conditions [13]. Other studies used engineered bio-organic polymer scaffolds to create 3D systems which have yielded important new information regarding cell signaling, phenotypic changes, angiogenesis, chemo-resistance of cancer cells and interaction of cancer cells with osteoblastic matrices [14–19]. Despite these advances, however, these latter bioengineered 3D-constructs do not reflect the *in vivo* conditions of live bone. This is because there is no well organized multicellular cell population consisting of stem/progenitor cells such as those related to mesenchymal, hematopoeitic and stromal origin that can respond to the presence of cancer cells and in turn have dual-feedback communications. Furthermore, there is no true bone matrix which contains embedded osteocyte cells and ECM that has well organized structural components such as collagen and many other proteins that generate architectural integrity. These ECM components together with the multicellular "periosteal and endosteal" layers of real bone play critical roles in assisting cancer cell attachment, proliferation, cell signaling processes and bi-directional communications between cancer and bone cells.

We have developed and utilized a roller tube model system which simulates closely the *in vivo* tissue under defined conditions and specific bone remodeling stages, i.e., resorption or formation, including hypoxic conditions which both solid tumors and bone experience naturally. This current report describes the development and multi-faceted application of this model for studies of cancer-bone metastasis/interactions.

2. Materials and methods

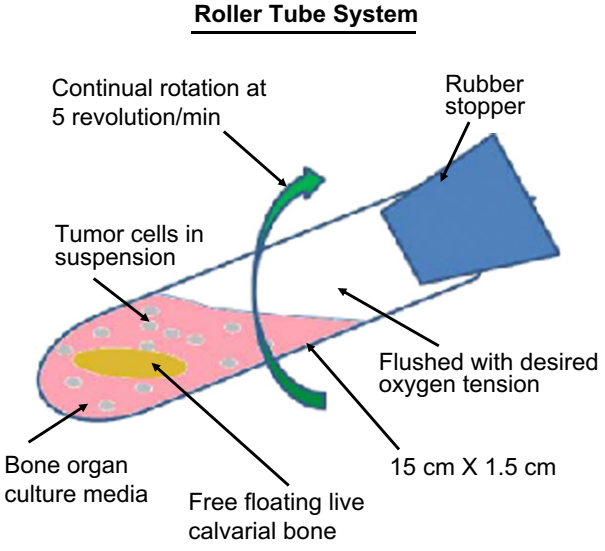
2.1. Roller tube system for calvarial bone organ cultures and cancer cell studies under hypoxic conditions and in the absence of immuno-reaction

Calvaria from 5–7 day old neonatal CD-1 mice (Charles River Laboratories, MA) were dissected under sterile conditions. Calvaria were cut in the occipital lobe, and partially in the frontal lobe, to produce a trapezoid structure and rinsed in culture medium. The live bone organ culture medium consisted of DMEM supplemented with bovine serum albumin (BSA), fraction V (5 mg/ml, Sigma Co.), 100 U/ml penicillin and 100 μg/ml streptomycin and 250 ng/ml amphotericin B (Gibco, Grand Island, NY) with "no" fetal calf serum (FCS) or ascorbate. The approach permits studies to be performed with remodeling events dissociated whereby when stimulated by inclusion of parathyroid hormone (PTH) [20] or PGE2 or conditioned media from cancer cells, calvarial bone undergoes predominantly osteoclastic bone resorption. For the bone formation model in addition to the above conditions, 150 μg/ml of sodium ascorbate was included in the bone culture media. In this system for co-cultures, a single calvarium was placed in each roller tube (15 cm × 16 cm, Bellco, Inc. N.J.) containing 2 ml of bone culture media with respective cancer cells (5×10^5 per tube) in suspension. (a) For the bone resorption model the culture media contained no FCS or ascorbate. (b) For the bone formation model the culture media contained no FCS but sodium ascorbate was included. The media and the tubes were flushed with a gas mixture of 5% O_2, 5% CO_2, and 90% N_2, for 60 s, the roller tubes were sealed with a stopper, and placed horizontally in a roller tube apparatus (Bellco Biotechnology, Inc. Vineland, N.J.) that makes 5 revolutions per min. in a 37 °C incubator, see Scheme 1. For individual cultures of bone alone or cancer cells alone the appropriate media and a single respective specimen was used and tubes processed as above.

2.2. Effects of cancer cell conditioned media, added factors and drugs on calvarial bone cultures under hypoxic (5% O_2) conditions

Five separate samples (a–e) for each breast tumor cell lines, MCF-7 and MDA-MB-231, and prostate tumor cell lines, LnCap Clone FGC and PC-3 (American Type Culture Collection), were grown in Dulbecco's Modified Eagle Medium (DMEM) in the presence of Fetal Calf Serum (FCS) for breast tumor cells or Roswell Park Memorial Institute media (RPMI) for prostate tumor cells. Tumor cells (1×10^6 per tube) were incubated at 37 °C for 8 days, with each tumor cell type in five (a-e) separate sterile, rubber-stoppered roller tubes at 5% oxygen tension (a total of 20 samples), see also Scheme 1. All tumor media was changed every 2–3 days, and freshly oxygenated with 5% oxygen in gas phase.

On day 6, media was changed for the second time, and all five (a-e) conditioned media for each of the four tumor types were collected for time point, T2, and stored at −20 °C. On day 8 the conditioned media were also collected, time point T3, and the roller tube experiment was terminated. T2 and T3 time point conditioned tumor media were combined for each of the individual a, b, c, d, and e-samples. These five different tumor conditioned media obtained for each of the different tumors were used to spike at 25% level the calvarial bone culture media. Conditioned media from four different human tumor cell lines grown under hypoxia were used to test their effects on live mouse calvarial bone cultures under conditions primed to undergo only bone resorption if presented with appropriate external stimuli. A single calvarium and 2.0 ml of bone culture media spiked with 25% of tumor media was added to each roller tube and incubated as described above. A total of 20 calvaria were used to set up one calvarium per conditioned cancer cell media from each of four cancer cell lines. A second set of such 20 calvaria with the same cancer cell media was set up but in the presence of 5 μM Indomethacin. A set of control calvarial bone cultures were carried out using: (a) five individual calvarial bone cultures with no cancer media addition, and (b) five calvarial bone cultured in the presence of added 20 nM PGE2 (Cayman Chemical Co., Ann Arbor, Michigan U.S.A.). These bone cultures were grown for 7 days with media changed every 2–3 days and the used media were stored at −20 °C. At the end of 7 day culture the calvarial bones were fixed and stained with H & E for histological analysis and the media were analyzed for prostaglandin E2 (PGE2) levels using commercially available PGE2 ELISA Kit as described in the protocol (Cayman Chemical Co., Ann Arbor, Michigan U.S.A.).



Scheme 1. The roller tube system was comprised of pyrex tubes of 15 cm × 1.6 cm with 2 ml of culture media, one free-floating calvarial bone and cancer cells (5×10^5) flushed with a mixture of 5% O_2, 5% CO_2 and 90% N_2 (using a gas cylinder ordered to specification, Medical Gas Supplier, Inc.) for hypoxic conditions. The tubes with sterile stoppers were placed horizontally in a roller tube apparatus with a setting of 5 revolutions per min in a 37 °C incubator.