In Vitro Production of Circulating Tumor Cells (CTCs) Using 3D Cultures of Human Tumor Tissues and Established Tumor Cell Lines

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Abstract

The ability to produce and collect Circulating Tumor Cells (CTCs) from cultured metastatic tumor tissue and established pancreatic cancer cell lines using a 3D perfusion culture system is demonstrated. The culture system used, the RealBio D⁴ ™ Culture System, incorporates low-sheer, tangential flow of nutrient medium above and below an open synthetic 3D cell scaffold, and gas-permeable membranes above and below the nutrient flow compartments to facilitate creation of *in vivo*-like gradients of nutrients, growth factors, and gasses that promote the recreation of an *in vivo*-like environment. This design allows CTCs generated within the cultures to migrate out of the cultured tissue or cell mass into the circulating nutrient medium in a manner reminiscent of the migration of CTCs out of tumors and into the blood stream *in vivo*. Once in the medium, the CTCs can be easily collected for characterization and further study.

Human metastatic pancreatic (to liver) tumor tissue from a mouse xenograft, and established human pancreatic cancer cell lines were cultured in the RealBio D⁴™ Culture System for at least 30 days. Cells migrating from the cultured tissues into the circulating culture medium were collected periodically throughout the study and characterized with respect to functional and cell surface CTC markers using a commercially available CTC technology designed for detecting CTCs in whole human blood (Vita-Assay™, Vitatex Inc., Stony Brook, NY). CTC's (including Circulating Tumor Progenitor Cells, CTPCs) were positively identified in the circulating medium at all time points examined.

CTCs were produced by the cultured primary tumor tissue at a rate of more than 100 CTCs per culture per day after 30 days accounting for nearly 10% of all viable cells shed into the medium. Highly metastatic cell lines (MIA PaCa-2 and AsPC-1) produced as many as 6,000 CTCs per day accounting for 22% of the viable cells migrating from the cultures. In contrast, poorly metastatic cell lines (PL45 and Capan-2) produced no more than 20 CTCs per day representing no more than about 3% of the viable cells.

The results presented here demonstrate that CTCs can be produced *in vitro* by heterogeneous tumor tissue cultures. The results also show that CTCs can be produced in large numbers by highly metastatic cancer cell lines grown *in vitro*, but that poorly metastatic cancer cell lines produce far fewer CTCs/CTPCs when cultured under the same conditions. The methods described here represent a valuable new approach for the study of CTCs *in vitro* with direct applicability for developing improved diagnostic assays for CTCs, the study of factors that affect their formation and migration from tumor tissue, and development of therapies targeting CTCs

Introduction

Circulating Tumor Cells (CTCs) have become a primary focus of many oncology researchers seeking a better understanding of the processes of cancer growth and metastasis. These rare cells have been detected in the blood of patients with a wide range of cancers and are thought by many to represent new targets for improved cancer diagnosis and treatment. Much about CTCs remains unknown including what genomic and/or physiological factors affect the generation and release of CTCs by a given tumor, though it has been theorized that hypoxia, nutrient starvation, and acidosis may be important. Unfortunately, systematic studies of CTC formation and release by tumors *in vivo* are complicated by the low number of CTCs produced, the complexity of the milieu in which they must be detected (i.e., whole blood), and the difficulty in manipulating the tumor microenvironment in order to perform well-controlled studies.

Very little work has been reported in which *in vitro* models have been used to study the processes related to generation and release of CTCs. This may be because traditional *in vitro* models lack several critical features required for properly modeling tumor behavior. For example, cell line-based *in vitro* tumor models established in traditional 2D cultures fail to account for the importance of 3D cell morphologies and arrangements in modeling normal cellular function. In addition, most traditional *in vitro* tumor models, whether established using primary tumor cell populations or cell lines, generally lack the ability to properly mimic the *in vivo* tumor microenvironment (e.g., nutrient and gas gradients) in a manner sufficient to provide biorealistic tumor representations. In contrast to traditional *in vitro* tumor models, the RealBio D⁴ ™ Culture System has been shown to maintain heterogeneous primary tumor cell populations in a realistic 3D environment that is conducive to the release of CTC populations including Circulating Tumor Progenitor Cells (CTPCs).

The RealBio D⁴ ™ Culture System was designed to mimic the tumor microenvironment found *in vivo*. The open 3D culture scaffold within the system enables cultured cells to maintain natural 3-dimensional morphologies and to migrate throughout (and out of) the cultured tissue. In addition, the system enables the formation of nutrient and gas gradients reminiscent of those in natural tumors, such that heterogeneous tumor cell populations are supported. Together, these features provide unprecedented potential for modeling key components of tumor structure and behavior such as tumor initiation, expansion, and metastasis *in vitro* while manipulating a host of environmental conditions (e.g., hypoxia, acidosis, etc.).

The work described here represents an initial "proof of concept" effort to leverage the complimentary capabilities of the RealBio D⁴ ™ culture technology and existing CTC detection technologies to develop an *in vitro* system for production and collection of human CTCs for subsequent use in studying tumor metastasis, chemotherapeutic efficacy, and other biological processes involving CTCs.

Materials & Methods

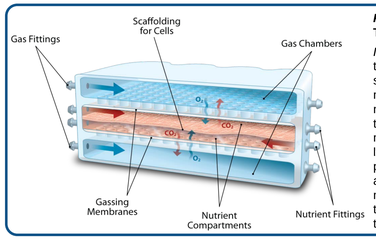


Figure 1.
The RealBio D⁴ ™ Culture Chamber.

In vivo-like conditions are promoted through the use of an open 3D cell scaffold and decoupled nutrient and metabolic gas supplies. Gradients of nutrients and gasses established across the cultured tissue mass promote varied microenvironments which in turn support long-term maintenance of mixed cell populations that mimic the composition and organization of natural tumors. CTCs migrate from tissue on the scaffold into the lower nutrient compartment where they are easily harvested for analysis.

3D Perfusion Tumor Cultures: Metastatic human pancreatic carcinoma tumor tissue (liver metastasis) was obtained as fresh mouse xenograft tumors (P0 or P1) from The Jackson Laboratory (Sacramento, CA). The tumor tissue was minced and partially digested using Liberase TM (Roche Applied Sciences, Indianapolis, IN) and DNase I before being triturated through successively smaller orifices until the resulting suspension could pass freely through a 16 g needle. A portion of the whole tumor cell suspension was infused into the top compartment of replicate RealBio D⁴ ™ Culture Systems primed with IMDM + 10% FBS and 5 mcg/mL gentamicin. The seeded culture systems were placed at 37^0 C with 5% CO_2 for 24 h to allow cells to settle onto and into the culture scaffold. After a 24 h settling period with no medium flow, the culture chambers were placed on a 45^0 incline and pulsed medium flow was initiated and maintained for the duration of each study (1 mL/min for 1 minute, 45 seconds every 30 minutes). Samples were collected from the bottom compartment of each culture chamber 3 times per week for analysis of glucose and lactate concentration and CTCs. Partial exchange of the circulating medium in each system was performed 1-3 times per week in order to replenish nutrients and remove waste products.

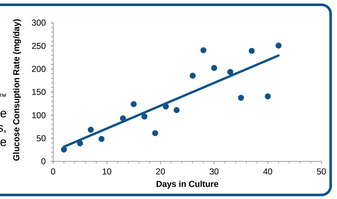
3D Perfusion Cultures of Tumor Cell Lines: Two human pancreatic cancer cell lines reported to be highly metastatic in mouse xenograft models (MIA PaCa-2 and AsPC-1) and two human pancreatic cancer cell lines that rarely metastasize in mouse xenograft models (PL45 and Capan-2) were obtained from the American Type Culture Collection (ATCC) and maintained in T-75 flasks in ATCC-recommended medium. Perfusion cultures were established for each cell line by infusing 2-3x10^6 cells into the top compartment of replicate RealBio D⁴ ™ Culture Systems primed with the appropriate media. The cultures were then maintained as described above for tumor cultures.

Collection and Characterization of CTCs: Samples of blood from the mice bearing the tumors used to initiate the 3D tumor cultures and medium samples collected periodically from the lower compartment of the culture chambers were analyzed for CTCs and CTPCs using the Vita-Assay™ AR6W platform. For all samples, viable CTCs were identified by their ability to bind to and internalize Vitatex's proprietary fluorescent Cell Adhesion Matrix (CAM) while also exhibiting positive staining with standard epithelial (Epi) markers for human pancreatic tumor cells (EpCAM and CA19-9). Viable CTPCs were identified in a similar fashion except that tumor progenitor markers (CD44v6 and seprase) were used in place of epithelial markers. All cells were also stained with the fluorescent nucleic acid dye Hoechst 33342 to aid in differentiating cells from cellular debris. Cells exhibiting positive staining with the various markers were counted manually under multi-parametric fluorescence microscopy.

Results

Figure 2.
Glucose Consumption for a Primary Pancreatic Tumor Culture in 3D Perfusion Cultures.

Primary tumor tissue cultured in the RealBio D⁴ ™ Culture System exhibits increased glucose consumption over extended culture periods, reflecting continued tissue expansion without the need for subculturing.



Results

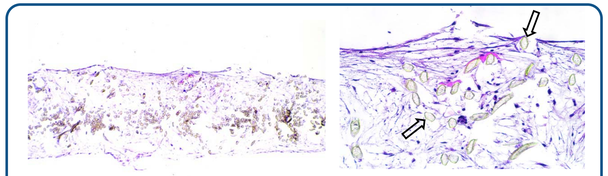


Figure 3.
H&E-Stained Sections of a 42-Day Old D⁴ Tumor Culture.

Light microscopy of embedded, sectioned, and H&E-stained scaffold from a 42-day old D⁴ culture of human metastatic pancreatic tumor tissue from mouse xenograft reveals 3D cell arrangements and infiltration of cells throughout the scaffold material (right). Higher magnification of the same section (right) shows cells distributed across open space within the scaffold and minimal association between cells with scaffold fibers (arrows). Note: scaffold fibers diameter = approximately 20 microns.

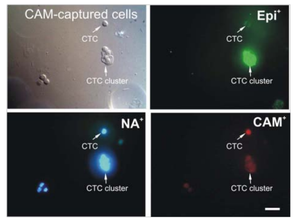


Figure 4.
Detection of CTCs and CTPCs.

Representative images for CTC identification using the Vita-Assay™ AR6W platform are shown. Cells captured by the Vitatex proprietary CAM (upper left) must exhibit positive staining for epithelial tumor marker (upper right) and internalized CAM (lower right) to be positively identified as a CTC. Data for CTPC identification is similar except that a tumor progenitor marker is substituted for the epithelial tumor marker. Nuclear staining (lower left) was used to aid in visualization of cells that did not stain positive for CTC and CTPC markers.

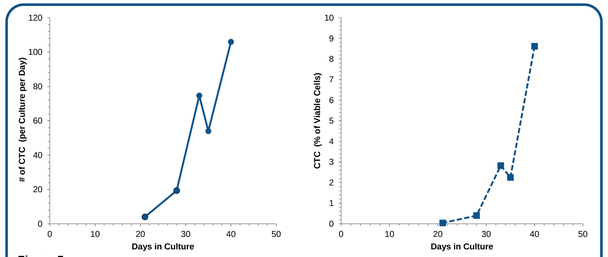


Figure 5.
Release of CTCs by D⁴ Culture of Metastatic Human Pancreatic Tumor Tissue.

CTCs present in the circulating medium collected from the bottom compartment of D⁴ culture chambers were identified using the Vita-Assay™ AR6W platform. The mean sum of CTC cell counts (excluding clusters) is shown in the left panel and normalized as a percentage of total viable cells in the right panel. For comparison, 30 CTCs were detected in 0.8 mL of whole blood from the mouse bearing the tumor used to initiate the cultures.

Results

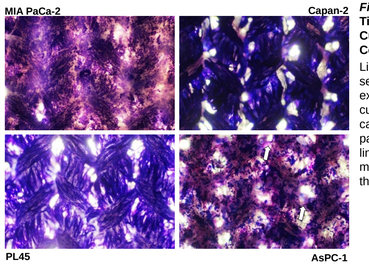


Figure 6.
Tissue Development in 13-Day Old D⁴ Cultures of Human Pancreatic Tumor Cell Lines.

Light microscopy of Hema 3-stained sections of culture scaffolds reveal the extent of cell coverage for 13-day old cultures from different human pancreatic cancer cell lines. The arrows in the panel corresponding to the AcPC-1 cell line highlight strands of extracellular matrix material that were found across the upper side of the culture scaffold.

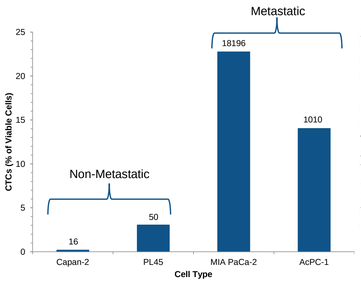


Figure 7.
Release of CTCs by D⁴ Cultures of Human Pancreatic Tumor Cell Lines.

CTCs present in the circulating medium collected from the bottom compartment of D⁴ culture chambers were identified using the Vita-Assay™ AR6W platform. The bars represent the CTC cell counts for a 3-day sampling interval of 25-day old cultures expressed as a percentage of total viable cells collected. The actual number of CTCs collected in each case is represented by the number above each bar.

Conclusions

- **CTCs may be produced *in vitro* by heterogeneous tumor tissue cultures maintained in the RealBio D⁴ ™ Culture System.**
- **CTCs may also be produced in large numbers by highly metastatic cancer cell lines grown the RealBio D⁴ ™ Culture System.**
- **Poorly metastatic cancer cell lines produced far fewer CTCs *in vitro* when compared *to* highly metastatic cell lines.**
- **CTCs produced *in vitro* can be detected and enumerated using existing technologies developed for detecting CTCs in human blood.**

Acknowledgements

The authors express sincere appreciation to Dr. Neal Goodwin at the Jackson Laboratory for facilitating access to the fresh xenograft tumor tissues used in this study and to Dr. Wen-Tien Chen and the staff at Vitatex Inc. for their assistance in performing CTC analyses.