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Assessing multiparametric drug response in tissue engineered tumor microenvironment models

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ABSTRACT

The tumor microenvironment is important in promoting treatment resistance of tumor cells via multiple mechanisms. However, studying this interaction often proves difficult. *In vivo* animal models are costly, time-consuming, and often fail to adequately predict human response to treatment. Conversely, testing drug response on human tumor cells *in vitro* in 2D cell culture excludes the important contribution of stromal cells and biophysical forces seen in the *in vivo* tumor microenvironment. Here, we present tissue-engineered models of both human brain and breast tumor microenvironments incorporating key stromal cell populations for assessing multiple mechanisms of therapeutic response using flow cytometry. We show our physiologically-relevant systems used to interrogate a variety of parameters associated with chemotherapeutic efficacy, including cell death, proliferation, drug uptake, and invasion of cancer and stromal cell populations. The use of flow cytometry allows for single cell, quantitative, and fast assessments of multiple outcomes affecting anti-tumor therapy failure. Our system can be modified to add and remove cellular components with ease, thereby enabling the study of individual cellular contributions in the tumor microenvironment. Together, our models and analysis methods illustrate the importance of developing fast, cost-effective, and reproducible methods to model complex human systems in a physiologically-relevant manner that may prove useful for drug screening efforts in the future.

1. Introduction

Precision medicine is gaining speed in development and clinical use. The use of screening technologies to assess therapeutic responses or predict outcomes in patient samples is important to developing new therapies and using appropriate and effective therapies in the clinic [1]. The ability to assess the response of a patient is imperative to increasing survival in diseases including fibrosis, cancer, and heart disease [2–4]. Recreation of tissues outside the patient body using tissue engineering methods offers the ability to potentially examine a patient's own tissues in a controlled setting [5,6]. These systems combine the benefits of mimicking tissue-level structures and interactions with the ease and manipulability of higher throughput screening platforms. Aside from precision medicine applications, they can also be used to test important scientific hypotheses related to disease related to the complex interactions that arise in a complete tissue and thus offer opportunities for drug discovery and development [7,8].

Basic *in vitro* tissue engineered models were first developed to examine the dynamics of cells within 3D microenvironments, offering one element of tissue-level complexity. It has been shown across multiple cell and tissue types that cells respond differently when moved from traditional 2D tissue culture to 3D culture with some sort of extracellular matrix [9,10]. Cellular exposure to chemical and physical cues in three dimensions has been linked to altered chemoresistance in tumor cells, differential changes to migration and invasion of normal and malignant cell types, altered cytokine expression, differentiation changes, and viability [11–13]. Tissue engineering provides a simplified platform for incorporating multiple cell types to study complex mechanisms. This platform has recently been applied to cancer research to study the complex tumor microenvironment, or tissue surrounding the cancer. Recent studies indicate the tumor microenvironment is important in promoting treatment resistance by increasing apoptosis resistance, proliferation, and invasion, as well as reducing drug transport to tumor cells [14,15]. Tissue engineered models can be an effective

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platform for simply incorporating multiple microenvironmental components to more accurately represent complex tumors and study therapeutic response of tumor cells.

Use of tissue engineered models has also allowed replacement of animal models and have offered not only the advantages of reduced animal use, but also many other benefits [16]. These include the ability to use human cells and patient-derived primary cells to more accurately represent human tissue without confounding species interactions [17]. Furthermore, inclusion of patient-derived primary cells paves technologies towards personalized medicine with the ability to incorporate patient cells into tissues recreated outside the patient body [18]. This leads to innovative drug screening platforms that can hopefully identify therapeutic regimens that can be truly successful for patients since they are identified using the patient's own cells.

Careful design and selection of components of the tumor microenvironment are important to the development of an appropriate platform for experimental use (Fig. 1). To use these systems, a careful balance between complexity and ease of use must be determined. Many factors within the tumor microenvironment can contribute to a tumor cell's behavior, however, incorporation of every element within the tissue would drastically reduce the ease of use of a system and can cause difficulties in outcome measures. Thus, careful formulation of the specific question, hypothesis, or objective should be considered before design of the system. This is followed by collection of relevant information to enable appropriate modeling either through literature or prior *in vivo* data. We recommend examining four key groups of factors within the design: Cells, Extracellular Matrix, Chemical & Physical Gradients, and Structures. The last component of design is the choice of outcome measures which can affect the timing, implementation, and specific cell culture conditions (culture vessel, imaging conditions, media preparations) that are used.

As tissue engineered models aim to mimic tissues, many techniques that are used *in vivo* can be translated to these *in vitro* models through careful planning and protocol development. We and others have demonstrated the use of standard histological techniques, intravital imaging, protein analysis, and gene expression analysis with tissue engineered models [19,20]. Specifically in cancer, these models can be useful for assessing outcomes related to chemotherapeutic, novel targeted therapeutic, and other therapeutic strategies in screening, discovery, and patient-specific regimen planning [21].

As one example, we have built tissue-engineered models (Fig. 2) of the complex region of the tumor-tissue interface to examine response of tumor cells to chemotherapy. Within these systems, we describe methods and results to examine multiple outcomes related to tumor malignancy, such as cell death and apoptosis, invasion, and drug uptake with chemotherapeutics. Briefly, we harvest tumor, stromal, and immune cells (Fig. 2A), label each with fluorescent organic dyes, incorporate the cells into an extracellular matrix and seed these into a tissue culture insert (Fig. 2B) with a 8 μm porous membrane through which cells can migrate to mimic invasion (Fig. 2C). Chemotherapeutics are flowed through the gel (Fig. 2D), and at the end of the experiment, the gels can be removed from the insert, the matrix is degraded leaving the cells for flow cytometry analysis (Fig. 2E). The membrane can be fixed and imaged via fluorescence microscopy to quantify percentage of cells migrated through porous membrane (%invasion) as described in our Methods section. With this strategy, we demonstrate how flow cytometry and imaging can be used to analyze several outcomes related to cancer malignancy in two distinct models of cancer (brain and breast) and how these models can be used to understand drug efficacy.

2. Material and methods

2.1. Cell culture

Human glioblastoma cell line U251 (generously provided by the Purow laboratory at the University of Virginia), HCC38, and HCC1806 (ATCC) were cultured in RPMI (Gibco) with 10% fetal bovine serum (FBS). Human primary astrocytes were purchased from Sciencell and cultured following manufacturer's recommendation. SV40-transduced human microglia were purchased from Applied Biological Materials and cultured in DMEM (Gibco) supplemented with 10% FBS. Human lymphatic endothelial cells (HMVEC-dLy, Lonza) were cultured in Endothelial Cell Growth Medium (EBM-2 basal media, Lonza) supplemented with recommended growth supplement kit (EGM-2MV BulletKit, Lonza). Human mammary fibroblasts (Sciencell) were cultured in according to manufacturer's instructions. All cell lines were grown sterilely in humidified atmosphere of 5% CO_2 and 95% oxygen at 37 °C. Cell lines were tested annually for mycoplasma (last test date: 12/2015, negative) and all experiments were completed afterwards.

2.2. 3D in vitro models of the tumor microenvironment

1. In a biosafety cabinet, seed endothelial cells in droplets on the underside of an 8 μm pore size 96-well tissue culture inserts (Corning).
   a. For brain studies: endothelial cells were not included so protocol begins at Step 4.
   b. For breast studies: 10,000 human lymphatic endothelial cells (LECs) were seeded in 25 μl droplets.
2. Allow to adhere for 2 h in incubator. Flip plate over to proper orientation. **Tip**: Ensure that cells do not become dehydrated by reapplying media as needed, checking every 30 min.
3. Maintain plates in a 37 °C, 5% CO_2 incubator for 48 h to allow endothelial cells to form a confluent monolayer, replenishing media in lower compartment as needed.
4. Label cells of interest with various Cell Tracker dyes (Life technologies) following manufacturer's recommended protocol. **Tip**: While each cell population can be labeled with a different cell tracker dye to specifically distinguish each population, flow cytometry gating and analysis can be simplified by labeling only the specific population of interest, (i.e. only tumor cells).
5. Incorporate cells into matrix (Fig. 2A). Total cellular concentrations range between 1 and 10 million cells/ml. Here, we used a total cellular concentration of 1 million cells/ml.
   a. For brain studies: U251 glioma cells, human astrocytes, and human microglia are homogenously resuspended in gel composed of 0.2% polyethylene glycol-diacrylate crosslinkable hyaluronan (ESI Bio) with 0.12% Rat Tail Collagen I (Corning) matrix [19].
   b. For breast studies: Human mammary fibroblasts and human breast tumor cells are homogenously resuspended in gel composed of 0.18 mg/mL Rat Tail Collagen I (Corning) with 0.5 mg/mL basement membrane extract (Trevigen).
6. Slowly pipette cell-gel solution into the upper compartment of 8 μm pore 96-well tissue culture inserts (Corning). **Tip**: Ensure there is a continuous interface between the gel solution and the insert edge to yield consistent results.
   a. For brain studies: 75 μl of cell-gel solution is added.
   b. For breast studies: 50 μl of cell-gel solution is added.
7. Allow solution to solidify in a 37 °C, 5% CO_2 incubator. (Fig. 2B)