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Embedded multicellular spheroids as a biomimetic 3D cancer model for evaluating drug and drug-device combinations

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ABSTRACT

Multicellular aggregates of cells, termed spheroids, are of interest for studying tumor behavior and for evaluating the response of pharmacologically active agents. Spheroids more faithfully reproduce the tumor macrostructure found *in vivo* compared to classical 2D monolayers. We present a method for embedding spheroids within collagen gels followed by quantitative and qualitative whole spheroid and single cell analyses enabling characterization over the length scales from molecular to macroscopic. Spheroid producing and embedding capabilities are demonstrated for U2OS and MDA-MB-231 cell lines, of osteosarcoma and breast adenocarcinoma origin, respectively. Finally, using the MDA-MB-231 tumor model, the chemotherapeutic response between paclitaxel delivery as a bolus dose, as practiced in the clinic, is compared to delivery within an expansile nanoparticle. The expansile nanoparticle delivery route provides a superior outcome and the results mirror those observed in a murine xenograft model. These findings highlight the synergistic beneficial results that may arise from the use of a drug delivery system, and the need to evaluate both drug candidates and delivery systems in the research and preclinical screening phases of a new cancer therapy development program.

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1. Introduction

Three dimensional (3D) *in vitro* cell culture models are being adopted as preclinical tools for studying tumor behavior and drug response [1]. This paradigm shift is in response to a growing body of evidence that 3D systems promote greater *in vivo*-like cell behavior than their two-dimensional (2D) counterparts due to recreating more of the characteristic traits of the native tumor environment [2,3]. As such, these models are proving more predictive than monolayer based systems. The majority of these 3D tumor cell models are prepared by either: (a) growing cells on non-adherent surfaces or in suspension to induce cell clustering; (b) seeding cells within a preformed polymer scaffold [4–7]; or (c) embedding cells within a hydrogel to promote cell cluster formation along with cell-matrix attachments [8,9].

With regards to the latter technique, several polymer compositions including Matrigel™ [10,11], collagen [12], and hyaluronic acid [13] are being used to create 3D scaffolds in an effort to recreate the native extracellular matrix (ECM)-like environments *in vitro* [14–17]. These systems promote differential cell behavior when compared to 2D systems, but fail to reproduce the tumor macrostructure found *in vivo* [3,18]. Clinical tumors usually consist of a singular structure with metabolically active cells at the surface and a necrotic core, while cell clusters in the 3D matrices are substantially smaller and numerous. Solid tumors also possess mass transport limitations stemming from decreased surface area-to-volume ratios and longer diffusion lengths, which are not present in single cells or small cell clusters [18,19].

To address these challenges, several methods of creating large cell clusters (>350 μm) are reported in the literature [20–22]. These techniques eliminate or minimize the surface attachment sites for cells, forcing them to interact principally with each other, and include spinning flasks, hanging drops, and agarose-coated plates. The resulting clusters, or spheroids, are of a similar size to small tumors. Unlike clinical tumors, they exist in an attachment-free microenvironment with very different mechanical and biochemical properties than the native ECM [23]. This is an important caveat

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to their use, as matrix attachments via integrins and substrate mechanics play crucial roles in cell differentiation and survival [24]. The interplay between the ECM and the tumor drastically affects drug response, epigenetic state, and metastasis in cancer [2,18]. Therefore, there is a need for additional methods to prepare stable and reproducible models which mimic the native tumor environment while being large enough for comparison to patient tumors.

In order to simultaneously study and model key cellular parameters that regulate form and function including cell adhesion, cell–ECM interaction, biochemical state, mechanical properties, and tumor macrostructure, we present a scalable and reproducible method for embedding and manipulating cancer cell spheroids inside a 3D collagen gel. It builds upon previous spheroid and spheroid-collagen models [25–30], and enables individual spheroid manipulation along with quantitative and qualitative whole spheroid and single cell analyses. Specifically, we describe the formation of human osteosarcoma and breast adenocarcinoma multicellular spheroids and subsequent embedding within a collagen matrix (Fig. 1). We hypothesize that a multicellular spheroid contained in an ECM derived matrix will respond differently to the first-line chemotherapeutic agent paclitaxel based on its delivery route in contrast to that observed in a 2D monolayer system. Herein, we report the effects of matrix stiffness, cell seeding number, cell type, and chemotherapeutic treatment on a collagen embedded spheroid.

2. Materials and methods

2.1. Cell culture

Experiments were performed on the pediatric osteosarcoma cell line U2OS and/or breast adenocarcinoma cell line MDA-MB-231 (ATCC, Manassas, VA). Both cell lines express high levels of E-Cadherin, readily form spheroids, and are well characterized, including their protein expression and secretion profiles as well as have been extensively studied in cancer research applications [12,31,32]. Cells were cultured in complete RPMI (U2OS) or DMEM (MDA-MB-231) media supplemented with 10% fetal calf serum and 1% penicillin-streptomycin solution (10,000 IU/mL penicillin; 10,000 μg/mL streptomycin). Cell cultures were maintained in 2D monolayers in a humidified incubator at 37 °C, 5% CO_2.

2.2. Spheroid formation

Cell aggregation was induced by growing cell suspensions in agarose-coated 96 well plates. Briefly, 1.5% (wt/vol) agarose solution was made by combining 0.15 g agarose (Sigma–Aldrich, St. Louis, MO) with 10 mL PBS. This solution was microwaved until agarose dissolution (~30 s) and kept on a hot plate during the well coating process, to prevent premature gelation. To prepare the 96-well plate, 70 μL of the hot agarose solution was pipetted into each well and allowed to cool for 20 min. Separately, cell monolayers were detached from their culture flask via a standard trypsinization protocol. Cells were counted and re-suspended in media to the desired concentration. Next, 100 μL of the cell suspension was added to each agarose-coated well. After 72 h, the resulting cell aggregates were lifted via gentle pipetting and were immediately seeded into collagen gels as described below.

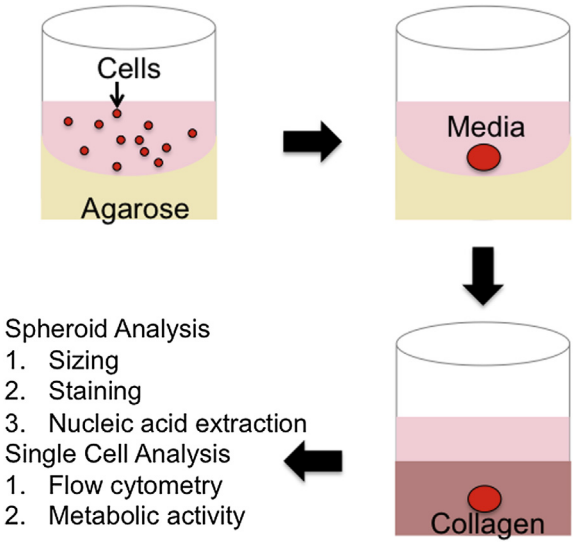

Fig. 1. Creation of Embedded Spheroids: Spheroid formation is encouraged by placing a suspension of cells (red) in media (pink) on agarose (yellow) coated wells. After 72 h, a spheroid is formed, and then transferred into a collagen gel. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

2.3. Collagen embedding

Spheroids were transferred into an unpolymerized collagen gel following a published procedure [12]. High Concentration Rat Tail Type I collagen (BD Biosciences) was diluted to 10 mg/mL in 0.02 N acetic acid and combined 1:1 with a buffer solution (100 mM HEPES in 2× PBS, pH 7.3). This mixture was further diluted with PBS to the experimental collagen concentration (2–5 mg/mL). This solution self-polymerizes into a gel after 1 h at 37 °C. Tumor cell spheroids were added to 100 uL of unpolymerized collagen solution in each well of a 96-well plate. After 1 h, 100 uL of media or media with drug was added on top of the gel. This media was removed and replaced every 24 h.

2.4. Tracking spheroid growth

After collagen embedding, spheroids were imaged every 24 h for the duration of the experiment. Images were acquired on a DMI600B microscope (Leica, Solms, Germany) with an ImagEM EM-CCD Camera (Hamamatsu Photonics, Hamamatsu, Japan) in a spinning disc confocal setup (Yokogawa, Tokyo, Japan). Imaging was done using Micro-Manager 1.4 Software (http://www.micro-manager.org). Resulting images were analyzed with ImageJ software to measure spheroid diameter.

2.5. Disaggregation of spheroids for assessment of metabolic activity

MDA-MB-231 spheroids were transferred into collagen (4 mg/mL) for 24 h prior to disaggregation by collagenase I treatment (2.38 mg/mL) (Life Technologies, Carlsbad, CA) in Hank's Buffered Salt Solution at 37 °C for 1 h. Disaggregated cells from both spheroids and monolayer culture were seeded for 12 h in 96 well plates. Cell viability was assessed via the MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) cell proliferation assay (Sigma, St. Louis, MO). Metabolic activity was calculated as the percentage of the positive control absorbance at 490 nm.

2.6. Live/Dead staining

MDA-MB-231 spheroids were stained with Calcein AM and Ethidium homodimer-1 (Molecular Probes, Eugene, OR), for live and dead cells, respectively 72 h after transferring to collagen (4 mg/mL). Spheroids were either imaged as previously described with the addition of laser excitation of the sample at 488 and 561 nm or disaggregated for fluorescent activated cell sorting (FACS). In addition to spheroids, MDA-MB-231 monolayers were stained for use as live and dead (after treatment with 70% methanol) controls. Cells were fixed with 4% formaldehyde prior to FACS with appropriate filters. Data was analyzed with FlowJo (Tree Star, Ashland, OR). Control samples were gated for live and dead populations, and then applied to the spheroid sample to calculate a percent alive and dead.

2.6.1. Nucleic acid extraction

Nucleic acid was extracted from fully embedded spheroid samples using the Trizol Reagent without additional purification or disaggregation (Life Technologies, Carlsbad, CA). Subsequent RNA concentration was then measured with a NanoDrop Lite (Thermo Scientific, Wilmington, DE). Ten MDA-MB-231 spheroids (20,000 cells each) yielded 4.735 μg RNA.

2.6.2. Nanoparticle preparation

Paclitaxel-loaded expansile nanoparticles (Pax-eNP) were prepared as previously described [33]. Briefly an oil-in-water mini-emulsion technique was used to polymerize monomer (5-methyl-2-(2,4,6-trimethoxyphenyl)-1,3-dioxan-5-yl) methyl methacrylate with crosslinker, 1,4-phenylene bis(2-methylacrylate) and the addition of (5% wt/wt) paclitaxel (MP Biomedicals, Solon, OH). A non-loaded fluorescently labeled eNP was made in a similar manner using a covalently incorporated rhodamine co-monomer.

Paclitaxel was selected for study because of its clinical use in the treatment of metastatic breast cancer and other solid tumor malignancies [34]. The mechanism of action is through binding and subsequent stabilization of microtubules which prevents dynamic reorganization [35]. Due to insolubility the compound is clinically dissolved in Cremophor El (1:1 solution of ethanol and polyoxyethylated castor oil) for *in vivo* administration [36].

2.7. Spheroid treatment with different drug delivery methods

MDA-MB-231 spheroids were made with 20,000 cells and subsequently embedded in collagen gels (4 mg/mL) for 24 h. Spheroids were exposed to either 100 ng/mL or 1000 ng/mL paclitaxel delivered via bolus dose (paclitaxel cremophor/ethanol) in the media or via Pax-eNP. In both cases, cells were incubated with the drug treatment for 24 h with monitoring of spheroid size over the following week.

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