Continuously perfused microbubble array for 3D tumor spheroid model

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Multi-cellular tumor spheroids (MCTSs) have been established as a 3D physiologically relevant tumor model for drug testing in cancer research. However, it is difficult to control the MCTS testing parameters and the entire process is time-consuming and expensive. To overcome these limitations, we developed a simple microfluidic system using polydimethylsiloxane (PDMS) microbubbles to culture tumor spheroids under physiological flow. The flow characteristics such as stream-line directions, shear stress profile, and velocity profile inside the microfluidic system were first examined computationally using a COMSOL simulation. Colo205 tumor spheroids were created by a modified hanging drop method and maintained inside PDMS microbubble cavities in perfusion culture. Cell viability inside the microbubbles was examined by live cell staining and confocal imaging. E-selectin mediated cell sorting of Colo205 and MDA-MB-231 cell lines on functionalized microbubble and PDMS surfaces was achieved. Finally, to validate this microfluidic system for drug screening purposes, the toxicity of the anti-cancer drug, doxorubicin, on Colo205 cells in spheroids was tested and compared to cells in 2D culture. Colo205 spheroids cultured in flow showed a threefold increase in resistance to doxorubicin compared to Colo205 monolayer cells cultured under static conditions, consistent with the resistance observed previously in other MCTS models. The advantages presented by our microfluidic system, such as the ability to control the size uniformity of the spheroids and to perform real-time imaging on cells in the growth platform, show potential for high throughput drug screening development. © 2011 American Institute of Physics. [doi:10.1063/1.3596530]

I. INTRODUCTION

Considering the rising costs of developing new anti-cancer therapies, it is of prime importance to develop efficient, inexpensive, and high throughput anti-cancer therapy testing systems, which more realistically mimic complex *in vivo* solid tumor pathophysiological conditions. The conventional method to screen therapies and drug targets is to first culture the tumor cells in suspension and/or monolayers. However, recent work has shown that many new therapies and drug targets lose their efficacy when applied in 3D tissue environments, despite their promising results when tested on tumor cells cultured with conventional methods.¹ Furthermore, certain potential targets exert their role only in complex tissue environments.^{2,3} Hence, the application of multi-cellular tumor spheroids (MCTSs) as a primary tool in anti-tumor drug development has been proposed by numerous researchers.⁴⁻⁹

MCTSs represent a complexity level beyond monolayers of cells in which hypoxic and necrotic cells are surrounded by highly aggressive, metastatic cells. This tumor spheroid structure more closely resembles *in vivo* tumors, which provides insights into the growth of tumors as well

as the complexity of the tumor microenvironment. To date, tumor spheroids have been used in cancer research for several decades and have contributed to our current understanding of cancer biology. They have been investigated in the contexts of experimental radiotherapy, photodynamic treatment, hyperthermia, and target specific chemotherapy and immunotherapy.^{10–14} Moreover, recent studies have shown that the gene expression profiles,^{15,16} growth kinetics and metabolic rates¹⁶ of MCTSs are similar to clinical conditions and hence, are believed to mimic *in vivo* solid tumors in their response to new drug targets and immunological factors. Therefore, MCTSs could help eliminate less efficient cancer drugs and therapies in earlier stages of testing before they enter expensive animal testing, and promote innovative drug targets that would otherwise fail in the traditional drug screening assays.

Commonly used culture techniques to create tumor spheroids include the hanging drop method,^{17,18} the liquid-overlay technique,¹⁹ and the gyratory rotation technique.²⁰ In the hanging drop method, spheroids are grown in droplets placed on an inverted microplate. In the liquid-overlay technique, cells are grown on culture plates covered with a thin film of agar, preventing cells from attaching to the surface so that individual cells can grow into aggregates or spheroids. In the gyratory rotation technique, trypsinized cells are cultured in a stirred flask, thus preventing them from adhering to the substrate and favoring cell-cell interactions. All these techniques have common disadvantages such as the formation of unequally sized spheroids, long cultivation times, inaccessibility for testing in the growth platforms, and difficulty to adapt to high throughput screening platforms. Current efforts to develop new systems are underway using modern micro-fabrication techniques to generate uniformly sized spheroids over shorter time frames.^{21–23} Our group recently reported the growth of homogenously sized 3D melanoma aggregates in microbubble compartment arrays²⁴ that were fabricated using polydimethylsiloxane (PDMS) gas expansion molding.²⁵

MCTSs are a frequently used *in vitro* model of avascular tumor growth and the physiological tumor microenvironment. Avascular tumors occur at early stages of tumor growth. The nutrients from the blood are obtained by the tumor mass only through its outer surface as blood vessels are not yet formed. Similar conditions occur again when the tumor progresses into a metastatic state where cells can escape and migrate to a new location.²⁶ The continuous perfusion culture enables one to recapitulate the tumor microenvironment with realistic concentration gradients and establish physiologically relevant drug profiles in the exposed spheroids.

In this study, we exploit the microbubble technology and develop a simple microfluidic device to maintain uniformly sized tumor spheroids in an array format. In this device, tumor spheroids are created and allowed to proliferate under flow condition, mimicking *in vivo* avascular tumor conditions and are accessible for imaging and analysis.

II. MATERIALS AND METHODS

A. Cell culture

Colon cancer cell line Colo205 (ATCC number CCL-222) and breast cancer cell line MDA-MB-231 (ATCC number HTB-26) were obtained from ATCC (Manassas, VA). Colo205 and MDA-MB-231 cell lines were maintained in RPMI 1640 (Sigma) and DMEM media, respectively, with 10% (v/v) fetal bovine serum and 100 U/mL Penicillin-streptomycin at 37 °C in a 5% CO₂ incubator.

B. Colo205 spheroids

Colo205 MCTS formation was initiated using a modified hanging drop method. Colo205 cells grown in culture flasks were trypsinized for 5 min and then resuspended in fresh RPMI 1640 media at a concentration of 1.5×10^6 cells/ml. An array of 5 μ l droplets of cell suspension was then placed on the lid of the 60 mm Petri dish. The lid with droplets was then inverted over the Petri dish containing 5 ml of RPMI medium. Aggregate formation was examined using bright field microscopy after incubating for 12 h. The formed spheroids were transferred by pipette and gently resuspended in 1X DPBS with Mg²⁺ and Ca²⁺ (Invitrogen) at a concentration of

2×10^5 cells/ml and used in cell capture experiments. Spheroid aggregates larger than $100 \mu\text{m}$ in diameter were subjected to gentle pipetting to generate smaller aggregates.

C. COMSOL Simulation

Before culturing cells inside the microfluidic system, a COMSOL simulation model was developed to understand the flow characteristics within the microbubbles under physiological shear flow. The Navier–Stokes equation with no slip boundary conditions was used to solve the model. A Newtonian fluid with density $1 \times 10^3 \text{ kg/m}^3$ and viscosity 0.01 Pa s was used to represent the flow buffer. Shear stresses in the range of $2\text{--}10 \text{ dyn/cm}^2$ were used for the COMSOL simulations and spheroid seeding into the microbubbles. Fluid was perfused through the microbubbles containing tumor spheroids at 0.6 dyn/cm^2 to mimic the fluid mechanical environment of the microcirculation and postcapillary venules.²⁷

D. Colo205 cell preparation for cell capture experiments

Colo205 cells were detached from tissue culture flasks using Accutase™(Sigma) and then incubated in fresh media at 37°C in a shaker up to 3 h for surface receptor recovery. The cells were then resuspended in 1X DPBS containing Mg^{2+} and Ca^{2+} to a concentration of 2×10^5 cells/ml. Cell viability of $>95\%$ was confirmed by the trypan blue exclusion test prior to each cell capture experiment.

E. PDMS microbubbles

Microbubbles in the size range of $200\text{--}500 \mu\text{m}$ in diameter with $50\text{--}200 \mu\text{m}$ top openings (in square, circular, and triangular shapes) were fabricated in PDMS using the gas expansion molding (GEM) technique described in detail elsewhere.²⁵ A vacuum-assisted coating (VAC) technique²⁵ was used to completely fill the microbubble with liquid and remove any trapped air bubbles. 1X DPBS was pipetted on top of the microbubbles to completely cover the top openings before placing the microbubbles inside a vacuum chamber. Negative pressure was applied for 30 min to draw out the air trapped inside the microbubbles therefore injecting PBS buffer into the microbubble cavities. The primed PDMS microbubbles were then used in the perfusion chamber assembly. The average size of the microbubbles used in this spheroid capturing experiment was $207 \pm 9.7 \mu\text{m}$ in diameter with $99 \pm 1.8 \mu\text{m}$ circular top openings. For each experiment, a microbubble array consisting of 25–30 microbubbles was used.

F. Perfusion chamber

The perfusion chamber was assembled by mounting a rectangular parallel plate flow chamber (Glycotech, Rockville, MD) on top of the PDMS microbubble array. A $125 \mu\text{m}$ thick rubber gasket was used to seal the device with the PDMS surface and create a rectangular path for the perfusion flow. To create a tight sealing and prevent any air bubbles from entering, the top plate with the gasket was connected to the vacuum pump by a Tygon tubing. The top of the parallel plate chamber is made of transparent polycarbonate that allows for easy imaging. Medical grade Tygon tubing was used to connect the chamber inlet to the cell suspension and the outlet to the syringe. Ethanol (70% v/v) was perfused through the chamber for 15 s to sterilize the tubing and flow chamber. Sterile 1X DPBS buffer was perfused to remove any residual ethanol prior to the cell capture experiments.

G. PDMS surface preparation

The PDMS surface was incubated with $0.5 \mu\text{g/mL}$ of recombinant E-selectin/Fc Chimera (R&D systems) in PBS for 45 min. To prevent nonspecific binding of cells, the surface was then incubated with 3% filter sterilized bovine serum albumin (BSA) for 90 min.

H. Cell capture inside PDMS microbubbles

PDMS surfaces were prepared as described above for both spheroid and single cell capture inside the microbubbles, mimicking the postcapillary venules where activated endothelial cells express selectins on their luminal surface. Colo205 cells were suspended in 1X DPBS and perfused through the parallel flow chamber at 2 dyn/cm² shear stress. Interactions between the E-selectin counter ligands expressed on the cell surface and immobilized E-selectin facilitate the tethering and rolling of the cells on functionalized PDMS surface. The rolling cells were trapped in the microbubble cavity as they approached the microbubble openings under flow conditions. The microfluidic system with cells trapped inside was then maintained at 37 °C in a 5% CO₂ incubator with perfusion of fresh RPMI media at 0.6 dyn/cm² shear stress.

E-selectin, a transmembrane glycoprotein expressed by endothelial cells upon inflammatory stimulation, is believed to play a major role in the metastasis of epithelial cancer cells.^{28,29} The interaction between E-selectin and its counter ligands expressed by circulating leukocytes and metastatic tumor cells leads to the tethering and rolling of these cells on the blood vessel wall.^{30,31} To successfully capture the Colo205 MCTSs under flow inside the microbubble cavity, E-selectin mediated ligand:receptor interactions were initiated to separate Colo205 spheroids and cells from MDA-MB-231 cells based on their differential adhesion phenotypes on functionalized microbubble and PDMS surfaces.

Before the cell capture experiments, the assembled apparatus was secured to the stage of an Olympus IX81 motorized inverted epifluorescence microscope (Olympus Americam Melville, NY). A CCD camera (Hitachi, Tokyo, Japan) and DVD recorder (Sony Electronics) were used to perform image capture for offline data analysis.

I. Cell staining and doxorubicin toxicity assay

Viable Colo205 and MDA-MB-231 cells were stained by incubation with 10 μM Celltracker Green and Celltracker Blue (Invitrogen) for 45 min at 37 °C. Cells were washed twice with PBS buffer to remove excess dye. The Celltracker probes stay within living cells through several generations and are inherited by daughter cells after cell fusion. Dead cell staining was performed using propidium iodide (PI) which intercalates into free double stranded nucleic acids in unhealthy cells. Doxorubicin was added to the RPMI culture medium at a concentration of 10 μM to test its toxicity on both monolayer and spheroid cultures.

J. Imaging and data analysis

Tumor cells and spheroids cultured inside the microbubbles were imaged using a Zeiss 710 laser scanning confocal microscope. A 25× lens was used to acquire confocal z-stacks of 50–200 μm in depth with a z-stack interval of approximately 2 μm. Images were uploaded to the Zen 2009 software (Carl Zeiss MicroImaging GmbH) for analysis. The number of cells within a spheroid mass was estimated from the volume of the 3D spheroid image reconstructed from z-stacks divided by the average volume of a tumor cell (1200 μm³).

III. RESULTS AND DISCUSSION

It is well understood that the tumor microenvironment plays a critical role in the tumor growth, progression, metastasis and drug resistance. Recent studies show that the mechanical microenvironment plays a major role in modulating tumor cell responses, tissue development, and maintenance.^{32,33} It has been shown that flow induced shear stress in tumor cells upregulate various key genes (cyclin B1 and p21^{CIP1}) and downregulate several other genes (such as cyclins A, D1, cyclin dependent protein kinases). In addition to creating physiological shear stresses inside the microbubbles, perfusion flow also establishes a physiologically relevant drug concentration profile to the exposed spheroids.

Figure 1 shows the streamline directions, shear stress profile, velocity profile, and complex flow paths created inside the microbubble by the perfusion flow. The fluid velocity decreases

FIG. 1. A COMSOL model was used to demonstrate the flow characteristics inside the PDMS microbubbles that were assembled within a Glyotech parallel plate flow chamber. Streamline side view (a) and top view (b) show the three-dimensional circular flow paths produced inside one microbubble. The COMSOL model slice plot (c) shows the flow velocity magnitude inside one microbubble. The velocity decreases sharply with distance from the bubble top opening. The shear stress plot (d) shows the shear stress magnitude exerted by the perfusion flow at the inner surface of the microbubble. COMSOL simulation conditions: Perfusion flow rate: 0.403 ml/min; fluid velocity: 2.12 cm/sec; shear stress at the PDMS surface: 10 dyn/cm²; 600 μ m diameter microbubble with circular 100 μ m top opening.

FIG. 2. (a) Sequential images of the microbeads trapped and circling inside the microbubble. Black arrows indicate the direction of the perfusion flow. Red and green arrows follow two different beads in their circular trajectories. These values are in agreement with the COMSOL simulation results (Fig 1c). (b) The circular paths taken by the two beads were highlighted using ImageJ software. (c) Schematic of the parallel plate flow chamber set up. (d) Micrograph of the experimental set up used for the spheroid perfusion culture including syringe pump, parallel flow chamber, and cell solution, (d3) Microdroplets placed on the petri dish lid for Colo205 spheroid formation.

FIG. 3. (a) Micrograph showing Colo205 cells rolling on the PDMS surface functionalized with E-selectin recombinant protein at a shear stress of 2 dyne/cm². The edges of the microbubble and its triangular top opening are outlined in green. (b) Higher magnification micrograph showing rolling cells entering the microbubble openings. (c1, c2, and c3) Micrographs showing Colo205 cells captured inside the microbubbles and cultured for two days under flow condition at 37 °C and 5% CO₂ at humidified conditions. (d1, d2, and d3) Micrographs showing Colo205 tumor spheroids cultured inside the microbubbles in 4X, 10X, and 20X, respectively.

sharply in magnitude with distance from the bubble top opening. To characterize the streamlines inside the microbubble and to determine the flow rate range at which the cells can be kept inside the bubble, 5.27 μm diameter polymer beads (Bangs Laboratories) were introduced into the microbubbles under perfusion flow. Here the consistent sizes of the beads made the characterization process simple and efficient. The beads were observed to travel slowly at the lower end of the circular motion and then gain momentum as they approach the bubble's top opening, as shown in Figs. 2(a) and 2(b). Cells and microbeads trapped inside the microbubble cavity were found to travel in circular trajectories and rarely escape from the bubble when the fluid velocity was less than 2.21 cm/s (10 dyn/cm² shear stress) at the bubble opening. The experimental system used for spheroid capture inside the microbubble cavity and perfusion culture is illustrated in Figs. 2(c) and 2(d).

Colo205 cells were captured inside the microbubble by rolling on the E-selectin functionalized planar PDMS surface of the microbubble via ligand:receptor interactions and fell into the microbubbles, as shown in Figs. 3(a) and 3(b). Captured Colo205 cells were cultured inside the microbubbles for 2 days to form loose aggregates (Fig. 3). This technique was also used to capture

FIG. 4. Top view (a1) and side view (a2) of a spheroid cultured inside a microbubble. The spheroids are pseudo-colored to indicate the distance from the microbubble bottom surface, (b) 3D image of the Colo205 spheroid cultured inside a microbubble by perfusion, stained with Propidium Iodide (PI) and imaged with confocal microscopy for the cell viability assay. Live cells were stained using Invitrogen Celltracker probe. (c) Viability of Colo205 spheroids cultured inside the microbubbles by perfusion culture as a function of seeding duration.

spheroids initiated by the hanging drop method inside the microbubbles followed by culture for up to 5 days with fresh RPMI media in perfusion. The sizes of the spheroids captured inside each bubble were controlled by the duration of the perfusion. This technique was also effective in controlling the spheroid size variability between microbubbles. In an experiment with 20 microbubbles, the average number of cells captured in each bubble was 95.8 ± 5.6 SD. The spheroids then formed inside each microbubble and proliferated. The fluid flow inside the bubble pushes the spheroids to the bottom and sides of the spheres resembling hemisphere-shaped spheroid clusters with raised edges.

Confocal images of the cultured spheroids were taken and pseudo-colored to indicate the distance from the cells to the microbubble bottom surface, as shown in Figs. 4(a1) and 4(a2). Dead cells inside the spheroids were counted using PI staining and 3D confocal imaging, shown in Fig. 4(b). Figure 4(c) quantifies the viability of cell spheroids cultured inside the microbubble system. A greater number of dead cells were detected at the bottom of the spheroids near the microbubble spheroid interface. Results from the toxicity assay of a common chemotherapy drug, doxorubicin ($10 \mu\text{M}$), on Colo205 cells cultured in a monolayer (Fig. 5(a)), Colo205 spheroids in static culture (Fig. 5(b)), and Colo205 spheroids cultured inside the microbubble perfusion culture (Fig.

FIG. 5. (a) Toxicity of doxorubicin drug on Colo205 cells cultured in monolayers. The percentage of cells viable after 48 h was $16.67\% \pm 1.49$ SD. (b) Doxorubicin toxicity on Colo205 spheroids cultured in agar-coated plates under static conditions. The percentage of cells viable after 48 h of treatment was $42.85\% \pm 3.97$ SD. (c) Doxorubicin toxicity on Colo205 spheroids captured and cultured inside microbubbles by perfusion flow. The plotted values are the average number of viable cells in each microbubble. Three microbubbles were used for the perfusion experiments and two microbubbles for the untreated group. The percentage of cells viable after 48 h of perfusion culture was $51.36\% \pm 1.93$ SD. Doxorubicin at $10 \mu\text{M}$ concentration was used in all three conditions.

5(c)) are demonstrated in Fig. 5. Colo205 spheroids show greater resistance to doxorubicin compared to the monolayer Colo205 cells, consistent with previous research on drug resistance of colon and breast spheroid cultures.^{34,35} This increased doxorubicin resistance in the spheroid cells may be due to cell-cell contact responses, internal protective mechanisms, and/or mass transfer limitations. Higher drug resistance in spheroids cultured inside the microbubble in perfusion flow compared to spheroids cultured in static suspension culture was also observed. The difference in the drug toxicity is likely due to limited exposure of cells to doxorubicin within the microbubbles, which may resemble the conditions prevailing in avascular tumor growth *in vivo*. The increased resistance demonstrated by spheroids in perfusion culture is similar to standard MCTS models studied by others.^{36–38}

Cells located at the top of the spheroids that are exposed to the media reflect the situation of actively growing tumor cells adjacent to capillaries *in vivo*. The cells at the microbubble bottom, on the other hand, become quiescent and undergo necrosis or apoptosis to form a secondary necrotic core. This arrangement of heterogeneous cell populations and the creation of pathophysiological gradients within the spheroids in microbubbles are similar to the micrometastases and avascular tumor microregions.

The advantages of the microbubble array technique include the ability to maintain the size uniformity of the spheroids and easy noninvasive testing during their growth. Attempts were also made to improve specific cell capturing efficiency of the microfluidic system. For instance, Colo205 cells were mixed with MDA-MB-231 cells that do not roll on the E-selectin coated PDMS surface in different ratios. The different adhesion phenotypes were used to sort the cells in the capture experiment (Fig. 6). Good enrichment of Colo205 cells was observed over the range of concentrations studied. The specific cell capture capability and easy control over the size unifor-

FIG. 6. Results of specific cell capture and enrichment study. Colo205 tumor cells stained with Invitrogen Cell Tracker Green were mixed with MDA-MB-231 tumor cells stained with Invitrogen Cell Tracker Blue at different ratios and used for cell rolling and capture inside the bubbles. MDA-MB-231 cells did not roll on E-selectin coated PDMS. The principal mechanism behind the enrichment is that the perfused cells rolling on the PDMS surface have an elevated probability of entering the bubbles compared to non-rolling cells.

mity of the spheroids make this model well suited for the development of co-culture spheroids. This separation process could be further improved through the optimization of the microbubble arrangements and opening shapes.

IV. CONCLUSIONS

A cost-effective 3D spheroid culture system which reflects the *in vivo* behavior of the cells in tumor tissues holds great potential for future drug development. We developed a simple microfluidic approach to culture tumor spheroids in perfusion culture that creates a 3D microenvironment, which resembles disease conditions. Colo205 spheroids created using the hanging drop method were captured and kept viable inside the microbubbles for 5 days with a continuous perfusion of fresh culture. Doxorubicin toxicity was compared among Colo205 monolayer cells, spheroids cultured in suspension culture under static conditions, and spheroids cultured in the microbubbles by perfusion culture. Greater resistance was observed in spheroids cultured in the bubbles, likely due to a combination of cell-cell contact responses, mass transfer limitations, and limited exposure of the cells to the drug. The microenvironment inside the microbubbles and flow conditions produced by the microfluidic system may resemble conditions prevailing in avascular tumor growth. The described technique is advantageous due to its low cost, ability to be automated and its usefulness for high throughput drug screening. Moreover, this model could be used to study therapeutic problems related to metabolic and proliferative gradients and 3D cell-cell and cell-matrix interactions. Further investigations on the enrichment of specific cell types and the capture of primary tumor cells within microbubbles to create spheroid co-cultures are currently under way.

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