PDMS Well Platform for Culturing Millimeter-Size Tumor Spheroids

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Abstract

Multicellular tumor spheroids are widely used as in vitro models for testing of anticancer drugs. The advantage of this approach is that it can predict the outcome of a drug treatment on human cancer cells in their natural three-dimensional environment without putting actual patients at risk. Several methods were utilized in the past to grow submillimeter-size tumor spheroids. However, these small models are not very useful for preclinical studies of tumor ablation where the goal is the complete destruction of tumors that can reach several centimeters in diameter in the human body. Here, we propose a PDMS well method for large tumor spheroid culture. Our experiments with HepG2 hepatic cancer cells show that three-dimensional aggregates of tumor cells with a volume as large as 44 mm^3 can be grown in cylindrical PDMS wells after the initial culture of tumor cells by the hanging drop method. This is a 350 times more than the maximum volume of tumor spheroids formed inside hanging drops (0.125 mm^3).

Keywords

three-dimensional cell culture, tumor spheroid, cell adhesion, soft lithography

Many types of tumor cells have a tendency to form multicellular aggregates of spheroidal shape when they grow under the conditions preventing significant adhesion of the cells to the walls of the culture vessel [1]. These aggregates, known as multicellular tumor spheroids (MCTSs), are a popular *in vitro* model for preclinical testing of anticancer therapy [2]. One of the standard techniques for MCTS culture is the hanging drop method in which tumor spheroids form at the bottom of up-ending droplets of cell growth medium [3]. This method produces MCTSs with the effective diameter of ~300 μm [4], two orders of magnitude less than the size of advanced tumors *in vivo* (> 3 cm) [5]. Small tumor spheroids are a useful model to study cellular response to chemotherapeutic drugs, but they cannot fully predict whether a specific cancer treatment is able to eradicate the tumor mass. Knowledge of this information is especially important for the development of optimized tumor ablation modalities [6]. While the preparation of tumor spheroids with the size of advanced tumors is out of reach because of the absence of vasculature in the spheroids, the limit in the spheroid size has not yet been reached.

The nature of tumor cells causes them to spread out and cover as much surface as possible, which is detrimental to spheroid formation. Several methods were suggested to prevent this spreading and thus force the cells to form three-dimensional aggregates. The growth of the tumor cell aggregates has been shown to correlate directly to the seeding density and initial size of tumor cells [7]. In some of the approaches, cell surface receptors were altered to allow less binding with surfaces and more binding between cells [8]. Others utilize non-adhesive surfaces [9] or porous scaffolds [10] to grow tumor cells into a desired three-dimensional shape. In the liquid overlay technique, tumor cells are placed in a gel composed of agarose or another biopolymer. The gel is fully submerged in a growth medium with nutrients that reach tumor cells through diffusion in the gel [11]. Cell spinning methods can result in larger spheroids (< 2 mm) than other

existing techniques can produce, but they require expensive equipment to prevent excessive shear to the cells [12].

Soft lithographic procedures have been utilized to create synthetic scaffolds for spheroid production [13, 14] or hemi-spherical "rifts" on microchannel walls [15]. The latter approach, referred to as a microbubble array, has been recently used to culture spheroids of colon cancer cells under physiological flow conditions [16]. The size of MCTSs produced by this method cannot exceed the rift diameter, which is 500 μm or less. In addition, the requirement for the cells to flow in the channels is detrimental to tumor generation. Other groups used polydimethylsiloxane (PDMS) to make micro wells, but due to the well size, it only allowed for small spheroid sizes [17]. One way to generate large tumor spheroids is to perfuse free floating cells together with rigid microspheres coated with cell adhesion molecules through a microchannel. Tumor cells attach to these particles and eventually form large spheroids [18]. The problem with this approach is that the core of such MCTSs is a synthetic structure but not a necrotic core as in natural tumors. This creates significantly different conditions for cell growth than the conditions existing in tumors *in vivo*.

In this paper, we used specially fabricated wells in which the substrate was divided into two parts: an adhesive center that served as an anchor for a spheroid and a non-adhesive periphery made of untreated PDMS that prevented cell spreading. We hypothesize that the initial clusters of tumor cells (prepared, for instance, by the hanging drop method) must be anchored to a surface to properly grow into a large-volume three-dimensional aggregate. It should be noted that the majority of tumor cells in the body are of epithelial origin (carcinoma). These cells grow in an anchorage-dependent manner, as shown previously [19-21]. Here, we report about the design

of the PDMS wells with different diameters using soft lithography and the growth of large tumor aggregates in the wells.

Human hepatocellular carcinoma cell line HepG2 was purchased from ATCC. They were grown under the 37°C 5% CO_2 conditions in T-75 culture flasks in Dulbecco's Modified Eagle's Medium supplemented with 10% fetal bovine serum and antibiotics/antimycotics (Invitrogen). When reaching more than 80% confluence, the cells were detached by using 0.05% trypsin (Invitrogen) and then re-suspended in a new flask. These procedures were repeated until the cells reached passage 4 or 5.

The hanging drop method [3, 4] was utilized to grow initial clusters of tumor cells. In this method, 35 µl drops of suspension culture with ~10^6 cells/ml were placed on the lid of a BD Falcon™ 24-well multiwell plate. The lid was then inverted and the drops were allowed to hang for several days. According to our measurements, the cluster size reached a saturated value of about 310 µm in three days (Fig. 1a). Figure 1(b) shows the top view of this cluster.

The clusters prepared by the hanging drop method were transferred to micro-centrifuge tubes via gentle suction by a pipette and centrifuged at 125×g for 5 minutes to ensure the strong attachment between cells. The pellets were then transferred to the PDMS wells (one hanging drop culture per one PDMS well) to further grow into larger aggregates. The centrifugation to pellet tumor cells has previously been used to generate initial tumor spheroids[11].

To prepare PDMS wells, 36 g of liquid PDMS (Skylgard 184 Silicone Elastomer Base) was mixed with 3.6 g (10% weight/weight) of Skylgard 184 Silicone Elastomer Curing Agent (Ellsworth Adhesives, Germantown, WI). The resulting solution was poured equally into each well of a 24 well plate with the insertion of metal rods of diameters 3, 5, and 8 mm as molds and

allowed to solidify in a Fisher Scientific Isotemp 500 series oven at 80°C for 3 hrs (Fig. 2). The final height of the wells was 9 mm. Cell pellets were placed into the PDMS wells (Fig. 2) and grew at 37°C in 5% CO_2 a New Brunswick Galaxy 48 R incubator for 14 days.

The growth medium was changed in the wells every day from day 1 to 10 and then every 12 hours after day 10. During this procedure, only the top layer (~80%) of the medium was removed to ensure tumor cells are submerged at all times. SYTOX green and C_{12} Resazurin red staining of cells was done afterwards to show both live (red) and dead cells (green) in the same sample.

The aggregate growth was measured by using a Nikon Eclipse TiS microscope equipped with a high-speed camera (QImaging Retiga EXi). Notches in the fine adjustment knob of the microscope were used as a ruler to measure the height of the aggregates, with each notch equal to 0.1 mm in height according to the manufacturer information. This method was verified by the height measurements from Z-stack images of tumor spheroids obtained using Olympus IX70 microscope (Olympus America, Inc., Melville, NY) coupled with a Photometrics CoolSNAP EZ camera (less than 10% difference between results gained between the z-stack and knob turning methods). The aggregate volume V was estimated from its height h and diameter d (measured from the bottom-view image of the spheroid) under the assumption the aggregate shape is a cylinder with a circular cross section: $V = \pi hd^2/4$. The true volume of the aggregate may be slightly less than the cylinder volume due to curvature of its top surface. However, this deviation is expected to be less than 33%, where the aggregate shape approaches a semi-ellipsoidal cap: $V = \pi hd^2/6$. At least four independent experiments per each diameter of the PDMS well were conducted. The data were plotted and statistically analyzed by using Prism 5.0.2 (GraphPad

Software). Statistical significance was determined by Student's *t*-test or one-way ANOVA with Bonferroni's multiple comparison test.

Figure 3(a, b) shows fluorescent images of HepG2 cell aggregate grown in 5 mm diameter PDMS wells for 5 days, with live/dead cell staining applied one hour before imaging. Additional images of HepG2 spheroids, cultured in PDMS wells for 7 days, are displayed in Figure 4. As seen in these images, HepG2 cells grow in PDMS wells as a single tightly-packed three-dimensional aggregate that has an outer layer of proliferating cells and an inner necrotic core, which is the structure typical for early-stage, avascular tumors in vivo. It is important to mention that the cells that put into PDMS wells without prior hanging drop culture form loose clusters without a three-dimensional structure (Fig. 3c). Similarly, HepG2 cell clusters do not grow into larger aggregates when they are not permitted to attach to the bottom of PDMS wells.

Figure 5 shows the effect of the PDMS well diameter on the volume of tumor cell aggregates. The maximum volume increased from 0.125 mm^3 at day 0 to the value between 10 mm^3 (in $\leq$3 mm wells) and 44 mm^3 (in $\geq$5 mm wells) at day 10. ANOVA shows statistically significant difference ($p < 0.0001$) in the aggregate volume between $\geq$5 mm wells and $\leq$3 mm wells. The effective diameter of the aggregates in 5 mm PDMS wells, calculated using the spherical volume formula, is around 2.19 mm. This is seven times more than the mean size of initial clusters prepared using the hanging drop method (0.31 mm).

In summary, we presented a simple method to culture millimeter-size three-dimensional aggregates of tumor cells using a PDMS scaffold. By using this PDMS well method, tumor aggregates grew to the maximum volume of 44 mm^3 in about 13 days (including 3 days of hanging drop culture), which was much higher than the volume of the spheroids obtained using

the handing drop method (~0.125 mm^3). The proposed technique can be further modified to grow bigger tumor cell aggregates by fabricating multi-level microchannels in PDMS to provide nutrients to tumor cells. These embedded microchannels can also be utilized to fine tune the aggregate shape. The design of such bioreactors for MCTS culture will be the focus of our future investigations and will eventually provide a three-dimensional *in vitro* tumor model for testing tumor ablation modalities and other methods for destruction of advanced and metastatic tumors.

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The authors declare that they have no conflict of interest.

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Figure legends

Figure 1: (*a*) Growth rate of HepG2 cell clusters during culture by the hanging drop method. Mean ± SD of six independent tests. Measurements were taken using Nikon Eclipse TiS microscope. (*b*) Top view of a HepG2 cell cluster (dark region) at the bottom of a hanging drop after three days in culture. Solid white lines are micrometer ticks that show that the effective diameter of this cluster is ~310 µm. 100X magnification. The image was taken using Nikon Coolpix 12 MP camera.

Figure 2: Three-dimensional culture of tumor aggregates in PDMS wells: the image of a PDMS well with a HepG2 aggregate (red) formed after five days in culture (top left), schematic of a PDMS well (middle and bottom left), and the image showing the aggregates growing in the wells of different diameter (right).

Figure 3: Tumor aggregate grown in 5 mm diameter PDMS wells for 5 days. (*a*) Image of an aggregate taken using a green filter. Bright green fluorescence indicates dead cells. (*b*) Overlay of aggregate images taken using green and red filters (green on top of red). Red fluorescence indicates live cells. (*c*) Without prior culture by the handing drop method, tumor cells do not form a three-dimensional compact aggregate in PDMS wells. Bar: 100 µm.

Figure 4: Gallery of HepG2 spheroids cultured in 5 mm diameter PDMS wells for 7 days. Shown are the overlays of spheroid images taken using red and green filters (red on top of green). The background of the images was darkened. Bar: 100 µm.

Figure 5: (a) Growth rate of HepG2 aggregates during culture in PDMS wells of different diameter. Mean of four independent tests. (*b*) Volume of HepG2 aggregates cultured in PDMS wells of different diameter for 10 days. The aggregate volume was calculated using the cylindrical model. Mean ± SD of four independent tests. * $p < 0.0001$.