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A multicellular 3D heterospheroid model of liver tumor and stromal cells in collagen gel for anti-cancer drug testing

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ABSTRACT

Two-dimensional (2D) monolayer cultures are the standard in vitro model for cancer research. However, they fail to recapitulate the three-dimensional (3D) environment and quickly lose their function. In this study, we developed a new 3D multicellular heterospheroid tumor model in a collagen hydrogel culture system that more closely mimics the in vivo tumor microenvironment for anti-cancer drug testing. Three aspects of cancer were chosen to be modeled based on their ability to resist anti-cancer drugs: 3D, multicellularity, and extracellular matrix (ECM) barrier. The hanging drop method and co-culture of liver carcinoma with stromal fibroblasts were used to form controlled and uniform heterospheroids. These heterospheroids were then encapsulated in collagen gel in order to create a 3D model of liver cancer that would act more similarly to in vivo ECM conditions. The 3D heterospheroid tumor model was tested with an anti-cancer drug to determine how each of the above aspects affects drug resistance. The results demonstrate that the 3D heterospheroid model is more resistant to drug over 2D monolayer and homospheroid cultures, indicating stromal fibroblasts and collagen hydrogel culture system provides more resistance to anti-cancer drug. This study will provide useful information toward the development of improved biomimetic tumor models in vitro for cancer research in pre-clinical drug development.

1. Introduction

Liver cancer is the fourth leading cause of cancer mortality in the world, leading to interest in developing cancer drugs with which to combat the disease. Unfortunately, cost hampers research. Drug development costs pharmaceutical companies hundreds of millions dollars a year. The cost of development for a single drug was 802 million dollars in the US in 2000 [1]. Part of this cost is related to animal upkeep during stage II drug development when testing for a drug's safety. Despite these costs, there is still no assurance that a drug proven safe on animals will also be safe on humans. Differences between the biochemistry of human beings and model organisms can result in differential drug reaction. An example is thalidomide which has no teratogenic effect in rats, however, is a potent teratogen in human beings [2]. Given past problems with the use of animals for the safe testing of drugs and other substances, researchers have been motivated to develop in vitro models for drug testing that will not only drive costs down, but will more accurately recapitulate human biochemistry and hence recapitulate human reactions to the drugs tested.

Two-dimensional (2D) monolayer cultures are the standard in vitro model, however, they fail to recapitulate the three-dimensional (3D) environment and quickly lose their function [3,4]. In order to improve monolayer cultures as model systems, monolayer cultures are layered on top of each other in order to produce a 3D effect. These layered monolayer cultures show improved function over their purely 2D monolayer counterparts [5]. Although, these sandwich cultures are useful for drug testing and toxicity, they do not recapitulate the architecture of bulk tumors. In bulk tumors, limited oxygen diffusion leads to hypoxia, which causes tumor resistance. As a result such experiments would show anti-cancer drugs to be more effective than they truly are.

Culturing hepatocytes into a 3D model can overcome these problems as they more closely recapitulate the dimensionality of the tumor microenvironment and thus should render results more similar to an in vivo tumor. A variety of 3D models exist that can be used for toxicity assays including hydrogels and porous membrane [6], however, the 3D model of spheroids best recapitulates attributes of the in vivo liver carcinoma and therefore best serves as a platform for liver cancer drug testing. Spheroids are aggregates of cells formed under non-adherent conditions and form a gradient of nutrients and gases. This gradient, particularly in regards to hypoxia, is important toward recapitulating the microenvironment of bulk tumor hepatocellular carcinoma as these bulk tumors are often starved for oxygen causing the continued differentiation and continued proliferation of surface hepatocytes [4]. This is evident by the behavior of the spheroids which are known to maintain

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properties particular to hepatocytes including urea production [7], albumin production, and cytochrome P450 activity when compared to monolayers [8,9]. Furthermore, these spheroids are known to resist certain anti-cancer drugs such as doxorubicin similar to in vivo conditions [10].

Although, many methods exist to form spheroids such as spinner flasks [11,12] and rotating wall bioreactor i.e. NASA bioreactor [3,13], these methods form heterospheroids that are inconsistent not only in size, but in the consistency of cell populations. The avidin–biotin method has the ability to produce heterospheroids with consistent cell population [14], however, the size of these heterospheroids remains inconsistent. Polystyrene dishes or concave microwell arrays coated with non-adherent surfaces have the ability to both create heterospheroids that are consistent in size as well as to create spheroids with consistent cell populations [15,16]. However, this would require acquiring a concave microwell array. The hanging drop method is able to overcome problems with inconsistent size, inconsistent cell populations, and lack of equipment. In the hanging drop method droplets of cells are plated and incubated on the lids of Petri dishes. Each droplet will form a singular spheroid from the cells contained within the droplet producing a spheroid with a consistent cell population. Furthermore, spheroid formation is initiated at about the same time, resulting in spheroids of consistent sizes. In addition, the hanging drop method utilizes supplies readily found around any scientific research lab and is relatively cheap.

Spheroids are able to recapitulate the dimensionality and hypoxia of the in vivo tumor microenvironment. However, spheroids composed only of parenchymal cells lack the stromal element. By adding stromal elements such as hepatic stellates, tumor associated macrophages, cancer associated fibroblasts, and other elements common to the in vivo liver carcinoma [17] to spheroids or other cultures, liver carcinoma viability can be improved. Cancer associated fibroblasts are able to release a variety of growth factors such as TGF-β and HGF and furthermore, aid in the metastatic process through the release of matrix metalloproteases [18]. In previous co-cultures, these cancer associated fibroblasts have been able to show the ability to prolong hepatic differentiation markers in culture, maintain enzymatic ability, and enhance proliferation [19]. In addition, cancer contains a thick barrier of extracellular matrix surrounding it which aids in liver cancer progression [20]. The cell-matrix provides a barrier to prevent the diffusion of drugs in the cancer and the body. Together, a more relevant tumor model would incorporate various aspects of cancer including (1) three-dimensions, (2) multiple cell types, and (3) an extracellular matrix barrier.

In this study, we developed a new 3D multicellular heterospheroid tumor model in a collagen hydrogel culture system that more closely mimics the in vivo tumor microenvironment for anti-cancer drug testing. The hanging drop method and co-culture of liver carcinoma with stromal fibroblasts were used to form controlled and uniform heterospheroids. These heterospheroids were then encapsulated in collagen gel in order to create a 3D model of liver cancer that would act more similarly to in vivo ECM conditions. The 3D heterospheroid tumor model was tested with an anti-cancer drug to determine how each of the above aspects affects drug resistance.

2. Materials and methods

2.1. Cell culture

Human hepatocellular liver carcinoma cell line (HepG2; gift from Dr. Charles Ross, Rutgers University) cells were cultured in DMEM High Glucose, 10% FBS (Biowest, Miami, FL), 2% penicillin and streptomycin (P/S, Gibco, Gaithersburgh, MD) and 2 mM L-glutamine (Gibco) at 37 °C and 10% CO_2. The medium was changed every 2–3 days. Murine stromal cells (3T3-J2 fibroblasts, purchased from Howard Green, Harvard Medical School, Boston, MA) were maintained in DMEM plus 10% FBS and 1% P/S. Prior to heterospheroid formation with HepG2, the confluent fibroblasts were growth-arrested by treatment with 12 μg/mL of mitomycin C (Sigma) for 2.5 h, as described [19]. After incubation, the cells were washed with PBS, trypsinized, and used for the heterospheroid formation.

2.2. Spheroid formation by hanging drop method

The hanging drop technique was used to agglomerate cells into spheroids. For homospheroid formation, a density of 1000 HepG2 cancer cells per 30 μL was obtained. For heterospheroid formation,

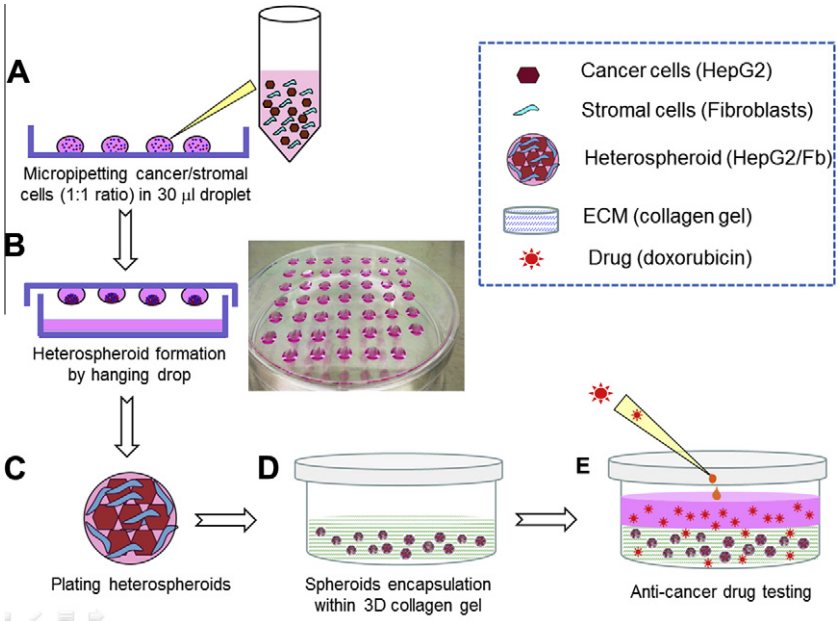

Fig. 1. Schematic diagram on the formation of heterospheroids by hanging drop method and 3D collagen gel culture for anti-cancer drug testing.

a density of 1000 HepG2 cells and 1000 growth-arrested fibroblasts per 30 μL was obtained. Using a multi-channel pipette, 30 μL of cell suspension was pipetted onto the lid of a 100-mm petri dish (Fig. 1A). Lid was flipped onto bottom dish to form droplets. The dish was filled with sterile deionized water to prevent the droplets from evaporating (Fig. 1B). Droplets were incubated for 2 days to form spheroids. Spheroids were then harvested for cell culture (Fig. 1C).

2.3. Spheroid diameter measurement

To examine the spheroid growth over time, spheroids obtained from the hanging drop method were transferred in a Petri dish for suspension culture. Spheroid images in suspension culture were captured over 5 days and analyzed by Sigma Scan Pro image software (SPSS, Inc, Chicago, IL) to measure the diameter.

2.4. 3D spheroid hydrogel culture system for anti-cancer drug treatment

For spheroid culture in collagen gel, approximately 50 spheroids were mixed with 0.3 mL collagen solution of 9 parts of type I rat tail collagen (1.2 mg/mL, BD Bioscience) and 1 part 10× DMEM. The mixed solution was added into 24-well and incubated for 1 h at 37 °C to form a collagen gel and spheroid encapsulation within the gel (Fig. 1D). After gelation, 0.5 mL of HepG2 culture medium was added and incubated at 37 °C and 10% CO_2 for anti-cancer drug testing (Fig. 1E). As a control in 2D monolayer culture, 50,000 HepG2 cells in 0.5 mL medium were seeded into 24-well plates. For anti-cancer drug treatment, 10 μM doxorubicin (Fisher Scientific) was added into the cell culture media (Fig. 1E).

2.5. Live/dead cell assay and cell tracker dye staining

Cell viability of the HepG2 homospheroids formed by the hanging drop method was examined by a Live/Dead cell assay kit (Invitrogen). 1 μM calcein-AM and 1 μM ethidium homodimer were added to the cell culture dish and incubated for 10 min at 37 °C. After washing with PBS, the cells were visualized with fluorescent microscopy (Nikon Ti–S). To examine initial cell distribution of HepG2 and fibroblasts within the heterospheroids, HepG2 were labeled with 1 μM 5-chloromethyl fluorescein diacetate (CMFDA, green cell tracker dye, Invitrogen) and fibroblasts were labeled with 1 μM CMTPX red cell tracker dye (Invitrogen) for 15 min prior to the spheroid formation by the hanging drop method. The spheroids were observed by the fluorescent microscopy.

2.6. Alamar blue (resazurin) assay

Alamar blue assay was performed to examine cytotoxicity and proliferation with and without anti-cancer drug treatment. 10 μM resazurin (Fisher) was added to 500 μL cell culture media in a 24-well plate and incubated at 37 °C for 1 h. The samples were collected every day and transferred to 96 well plates for the assay. After the sample collection, the cell culture plates were washed with PBS and fresh media with the anti-cancer drug was added to each well. Fluorescent intensity of the samples was measured using a fluorescent microplate reader (Gemini XPS, Molecular Devices) at wavelengths 530 nm for excitation and 590 nm for emission.

2.7. Cytochrome p450 drug metabolism function

Cytochrome P-450 A1 (CYPA1) enzymatic assay was performed by measuring the ethoxyresorufin-O-deethylase (EROD) activity. The EROD activity assay measures drug metabolism by measuring the ability of the HepG2 cells to convert ethoxyresorufin to resorufin. After 5 days of anti-cancer drug treatment, the cultures were induced to produce CYPA1 by 2 μM 3-methylcholanthren (3-MC, Sigma) for 48 h. The cells were then washed well with PBS, followed by 1 h incubation with 8 μM ethoxyresorufin (Sigma) in phenol red free culture medium at 37 °C. The sample medium was collected after incubation and the fluorescence intensity was measured at 530 nm excitation/580 nm emission wavelength by the fluorescence microplate reader. Resorufin (Sigma) standards at the range of 1–1000 nM were used to determine the sample concentration.

2.8. Statistical analysis

Data are expressed as the mean ± standard deviation (SD) from triplicate plates for each condition. Statistical significance was determined by a two-tailed Student's *t*-test ($^*P < 0.05$).

3. Results

Two types of spheroids were fabricated using the hanging drop technique: homospheroids and heterospheroids. Homospheroids are composed of one thousand HepG2 liver carcinoma cells each. Heterospheroids were composed of one thousand HepG2 liver carcinoma cells and one thousand stromal fibroblasts each. Live/dead cell staining was performed with calcein-AM and ethidium homodimer to examine the viability of the cells within the spheroids formed by the hanging drop method. Fig. 2A shows phase and fluorescent images of HepG2 homospheroids stained with the Live/Dead cell dyes. Highly compact cell aggregates were formed within the spheroids. Most of the cells were stained with green for live cells and only a few cells expressed red for dead cells, indicating good cell viability within spheroids.

To monitor HepG2 and fibroblasts within the heterospheroids, cells were labeled with two different fluorescent cell tracker dyes prior to spheroid formation and observed by fluorescent microscopy after spheroid formation by the hanging drop method. As shown in Fig. 2B, two cell types formed the heterospheroid that were spatially controlled by the hanging drop method, demonstrating homotypic and heterotypic cell–cell interactions between HepG2 and fibroblasts. Most of the growth-arrested stromal fibroblasts were observed in the center core of the spheroids, whereas HepG2 were observed both in the center core and at the spheroid periphery.

Spheroid diameters of homospheroids and heterospheroids were measured over 5 days using image analysis software. Uniform spheroids with diameters between 300 and 400 μm were obtained after 2 days of culture by the hanging drop method. After plating the spheroids in a Petri dish for suspension culture, the spheroid diameter initially decreased due to cellular reorganization. The spheroid diameter then increased continuously. The diameter increased by 35% for homospheroids and by 14% for heterospheroids at day 5. Homospheroids demonstrated higher spheroid growth rate compared to heterospheroids but there were no statistically significant differences over the 5 days of culture.

To examine cytotoxicity and proliferation of the spheroids for anti-cancer drug treatment, Alamar blue (resazurin) assay was performed by treating the cells with 10 μM doxorubicin. Fig. 3A exhibits a standard curve of HepG2 cells at various cell densities determined by the Alamar blue assay. There was a linear relationship between the fluorescence intensity and HepG2 cell number. Before testing anti-cancer drug treatment in the 3D spheroid hydrogel culture system, HepG2 cancer cells were tested in a 2D monolayer culture system as a control to investigate the effect of doxorubicin. Over the 4 days of the monolayer culture, treatment

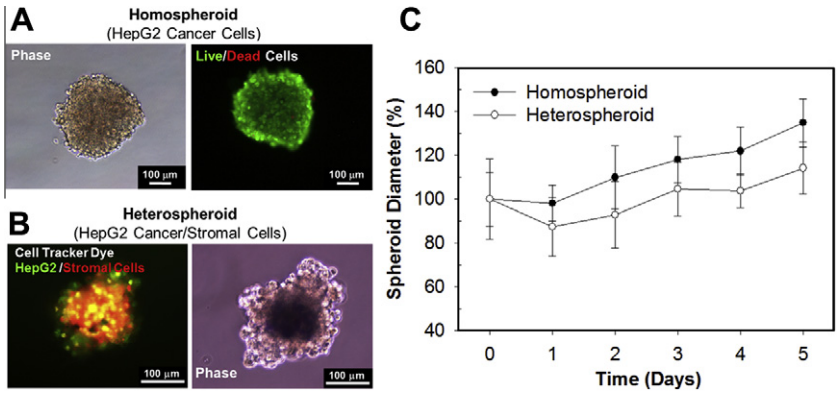

Fig. 2. Morphology and cell viability of homospheroids and heterospheroids formed by hanging drop method (day 4). (A) Live/dead cell assay of HepG2 homospheroid on day 4. (B) Image of HepG2/Stromal fibroblast heterospheroids stained with fluorescent cell tracker dyes (green, HepG2; red, stromal fibroblasts; yellow, overlap of green and red). (C) Spheroid growth (%) over time. The spheroid diameter over time was measured by the image analysis software and was normalized by the initial spheroid size formed by the hanging drop method for 2 days. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

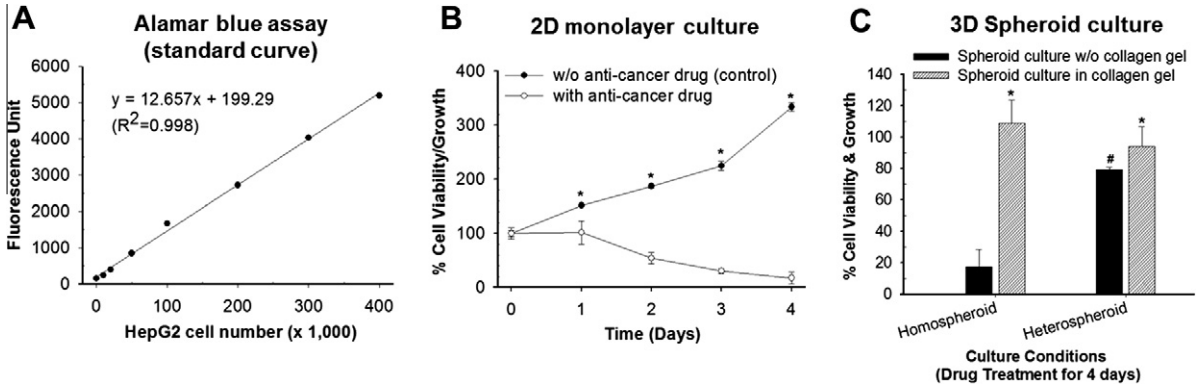

Fig. 3. Effect of anti-cancer drug (doxorubicin) on cell viability and growth in 2D monolayer and 3D spheroid cultures using Alamar blue assay. (A) Standard curve of HepG2 cells by the Alamar blue assay. (B) Cell viability/growth (%) in 2D monolayer cultures with and w/o drug (10 μM doxorubicin) treatment over 4 days. (C) Effect of anti-cancer drug (doxorubicin) on cell viability in spheroid cultures after 4 days of drug treatment. Cell viability and growth was assessed by the Alamar blue assay and normalized to values on day 0. Values represent averages ± SD ($n = 3$, $P < 0.05$, *spheroids in collagen gel vs. non-gel, #homospheroids non-gel vs. heterospheroid non-gel cultures). For heterospheroid formation, fibroblasts were growth-arrested by mitomycin C treatment prior to the spheroid formation.

with 10 μM doxorubicin resulted in 54% and 17% cell viability on days 2 and 4, respectively (Fig. 3B). By contrast, the HepG2 cells cultured without doxorubicin exhibited continuous increase in cell number during the culture period, demonstrating 334% cell growth (2.3-fold increase) after 4 days of culture. There were statistically significant differences between no drug and drug conditions on cell viability and proliferation at days 1–4.

Alamar blue assay in the 3D spheroid cultures reveals that 3D homo- and heterospheroid cultures in collagen gel showed significantly higher drug resistance to 2D cell cultures after 4 days of doxorubicin drug treatment (Fig. 3C). Homospheroids in collagen gel culture system showed more drug resistance than those not cultured in collagen gel. Similar results were observed in heterospheroid cultures. Among cell cultures without collagen gel, heterospheroid culture showed significantly higher drug resistance than homospheroids and 2D monolayer cultures but no significant difference in collagen gel cultures. Results of this study indicate that stromal fibroblasts and collagen hydrogel culture system provides more resistance to anti-cancer drug.

Fig. 4A exhibits cell viability and growth in the 3D heterospheroid hydrogel culture system with or without doxorubicin treatment for 4 days. 10 μM doxorubicin treatment resulted in 116% and 89% cell viability on days 2 and 4, indicating high drug resistance compared to 2D monolayer culture, as shown in Fig. 2B. For the heterospheroid hydrogel culture condition without doxorubicin, the cell number increased continuously throughout the culture period, showing 274% cell growth (1.7-fold increase) on day 4. Fig. 4B shows heterospheroid morphology cultured in collagen gel system with and without 10 μM doxorubicin treatment at day 4. The doxorubicin treatment for 4 days suppressed the spheroid growth and resulted in deterioration of spheroid structure and cell membranes, whereas the heterospheroids without the drug treatment retained good spheroid morphology.

Cytochrome P-450 A1 (CYPA1) enzymatic assay was performed to assess the drug metabolism function of HepG2 in 3D heterospheroid culture system with and without the doxorubicin treatment. The CYPA1 assay measures drug metabolism by measuring the ability of the HepG2 cells to convert ethoxyresorufin to resorufin. The assay reveals that heterospheroids in collagen gel showed significantly higher drug metabolism function than those without gel, indicating more drug resistance (Fig. 4C). The resorufin production rate of heterospheroid culture with 10 μM doxorubicin treatment was 0.047 ± 0.013 [nM/h] for collagen gel culture and 0.015 ± 0.002 [nM/h] for non-gel culture. Similar results were observed in the heterospheroid culture without the doxorubicin treatment.

4. Discussion

Spheroids have a variety of uses ranging from tissue engineering applications to assays for stem cells. These spheroids provide

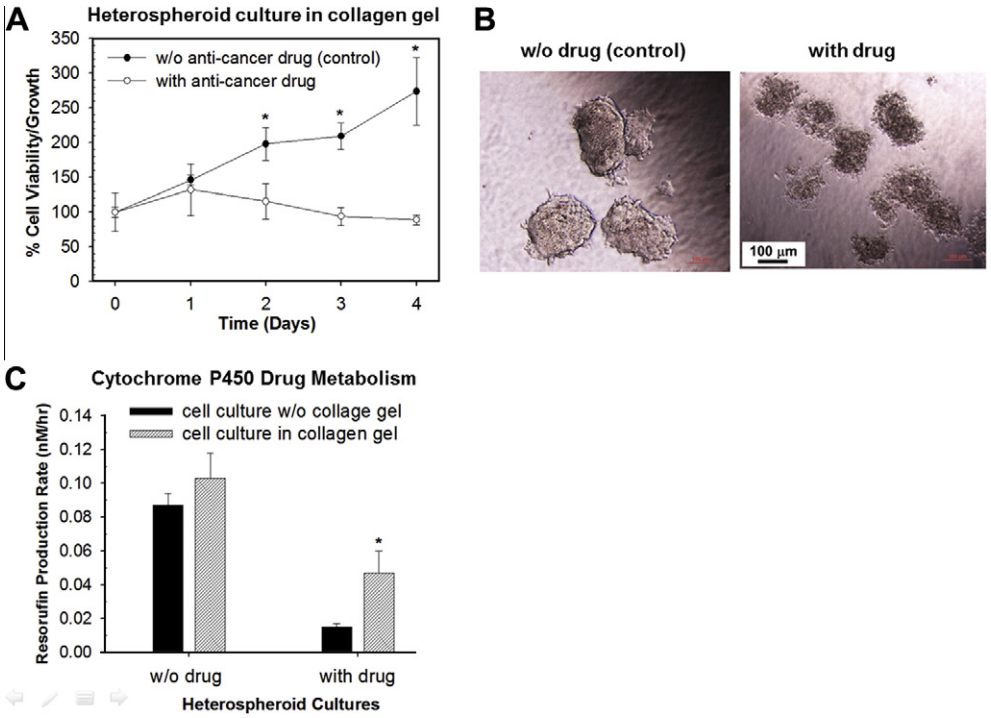

Fig. 4. Cell viability/growth and metabolic activity of 3D heterospheroids cultured in collagen gel with or w/o doxorubicin drug (10 μM) treatment. (A) Cell viability and growth (%) of the 3D heterospheroid cultures over 4 days. Values represent averages ± SD (n = 3, $^*P < 0.05$) (B) Morphology of heterospheroids (HepG2/Fibroblasts) with or w/o drug treatment for 4 days. (C) Cytochrome P450 drug metabolism assay (EROD activity) in the heterospheroid cultures with or w/o drug treatment (Day 7). Values represent averages ± SD (n = 3, $^*P < 0.05$, collagen gel vs. non-gel cultures).

a 3D environment which more accurately recapitulates the in vivo environment and thus were useful in this study of anti-cancer drugs on liver cancer. In this study, we have demonstrated a new 3D heterospheroid model of liver cancer and stromal cells in hydrogel culture system. The effect of the anti-cancer drug i.e. doxorubicin, on the spheroids was evaluated for spheroid viability, growth, and the cytochrome p450 drug metabolism function. The results of this study demonstrate that heterospheroid conditions with stromal fibroblasts exhibit enhanced resistance to drug over homospheroids and 2D monolayer cultures and that the collagen gel culture system was able to enhance the resistance of the spheroids to the drug. To our knowledge, this is the first report on the tumor model that incorporate various aspects of cancer including (1) three-dimensions, (2) multiple cell types, and (3) an extracellular matrix barrier.

To determine how dimensionality affects cell culture drug reaction, the proliferation rate of 2D i.e. tissue culture plates and 3D cultures i.e. multicellular tumor spheroid (MCTS) were compared. 3D cultures showed a greater resistance to the anti-cancer drug than 2D cultures as 3D cultures showed less of a decrease in proliferation than their 2D counterparts. These results are expected as the 3D cultures have an added dimension of cell–cell and cell-matrix contact which improves the viability of the system. Particularly, in regards to drug resistance, the spheroid has the ability to block the diffusion of the drug to all the cells as cells on the outer layers of the spheroid provide a natural barrier. Furthermore, the gradation of oxygen within the spheroid provides a hypoxic core similar to the hypoxic regions found within solid tumors. Hypoxia is known to modulate apoptosis, chemoresistance, and radioresistance in hepatocellular cancer [21]. Therefore, the hypoxic core of the spheroids should be able to modulate the cells such that they are able to resist the drug and thus show greater viability.

Besides, the spheroid itself, the collagen gel also serves as an ECM barrier to drug diffusion. This was evident in the collagen gel condition when drug reaction was measured between gel conditions and non-gel conditions. Gel conditions were able to show increased resistance compared to non-gel conditions, both in the Alamar blue assay and EROD assay as expected by previous studies in which ECM or other 3D substrate enveloped a spheroid showed similar improvement. This is expected not only because the ECM provides a barrier to drug diffusion, but also provides valuable cell-matrix contacts which aids in the viability and proliferation of the cells. However, whether resistance in collagen gel is the result of improved differentiation due to cell-matrix interaction between cells of the spheroid and the surrounding collagen gel or is due to improved proliferation of the cells within the MCTS is under question.

In this study, mitomycin C was used to treat fibroblasts in order to arrest their growth so that only proliferation of HepG2 in the cocultures could be measured. Mitomycin C is only supposed to arrest the growth of treated cells, however, also has the ability to affect other cells through the "bystander effect". The "bystander effect" refers to the ability of cells to kill other cells either through ionizing radiation or in this case chemical means. The bystander effect via ionizing radiation has long been known, however, the bystander effect via chemical means has been less studied. The exact mechanism of the chemical bystander effect has yet to be determined, however, it has been anticipated that mitomycin treated cells secrete Fas ligands which bind to Fas receptors of non-mitomycin treated cells to initiate apoptosis. Our preliminary results (unpublished data) show a decreased drug resistance of HepG2 cultures grown in media previously incubated with mitomycin treated fibroblasts showing a possible bystander effect. Future studies need to be done on the effect of mitomycin in 2D vs. 3D cocultures to determine if it is the bystander effect which may account for these results or whether it is some other effect such as synergy between mitomycin and doxorubicin or stiffness imposed by the fibroblasts on the spheroid.

In summary, we report a new 3D heterospheroid tumor model to mimic in vivo conditions. Results indicate the 3D heterospheroid tumor model in the hydrogel system is more resistant than 2D monolayer and homospheroid models. The 3D liver tumor model developed in this study can also be applicable to other cancer cell types (e.g. breast cancer and colon cancer cells) and other stromal cells (e.g. endothelial cells for vascularzed tumor model) for anti-cancer drug testing. This study will provide useful information toward the development of improved biomimetic tumor models in vitro for cancer research in pre-clinical drug development.

Acknowledgments

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