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Is 'Hanging Drop' a Useful Method to Form Spheroids of Jimt, Mcf-7, T-47d, Bt-474 That are Breast Cancer Cell Lines

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Abstract

Breast cancer is a type of cancer that has an extremely complicated structure. Recent studies have shown that breast cancer is common invasive cancer and unfortunately, its prevalence have been rising in women. Therefore, scientists use established cell lines in the laboratory for modelling and finding therapy. Spheroids which is known microtumors, it is well characterized models to mimic the natural environment. There are many devices which have been designed for forming spheroids. In this study, the 96-well hanging drop culture plate was chosen to form spheroids of the breast cancer cell lines JIMT, MCF-7, T-47D, BT474 at density of 2.5×10^4, 5×10^4, 7.5×10^4, 10^5 cells/well. Cells were imaged daily to check for aggregation and cell proliferation. Spheroid formation occured within 72 hours. The fluorescence microscope examination revealed that the morphological appearance of 3D spheroid was cell line dependent. In this study, more cells were used compare with the protocol that is given from manufacturer. The importance of our work is that spheroids were formed first time at high density.

Keywords: 3-D cell culture; Hanging drop; Spheroid; Microtumor; Breast cancer cell lines; JIMT; MCF-7; T-47D; BT-474

Abbreviations: ATCC: American Type Culture Collection; DMEM: Dulbecco's Modified Eagle's Medium; MEM: Minimum Essential Medium; RPMI-1640: Roswell Park Memorial Institute 1640 Medium; FBS: Fetal Bovine Serum; PBS: Phosphate Buffered Saline

Introduction

Breast cancer is the most common invasive cancer in women. According to World Health Organization (WHO), it is adversary affecting millions of women all over the world and the second main cause of cancer death in women, after lung cancer [1-16]. Due to the fact that breast cancer is a complex and heterogeneous disease, it is important to understand its mechanism. Thus, breast cancer is often modelled using established cell lines in the laboratory [6,7] BT-20 is the first breast cancer cell line that is established. After that, the MD Anderson series and MCF-7 were established cell lines which are commonly used for modelling in the laboratory. MDA-MB-435 was also characterised as a basal cell line [8]. Cancer cell lines: JIMT, MCF-7, T-47D, BT-474 are also widely used for modelling. Since their features, breast cancer cell lines became more widespread. For example: MCF-7 is a model cell line which could be used for researching hormone response [6].

Traditional cancer research uses two-dimensional (2D) cell culture methods. It contains cells on flat petri dishes that only allow moderate cell-to-cell contact. Due to the decreased cellular contact, 2D cell culture does not accurately represent tumor behavior [1-17]. Three-dimensional (3D) cell culture has advantages in providing more predictive data [3-11]. Since it enables greater contact between cells and the behavior of them is more reflective of *in vivo* cellular responses [9]. For example; epithelial cells normally exist as multilayer sheets *in vivo*, and when they were cultured on 3D system, they can act like a real tissue [5]. Chemical functionalities in 3D scaffolds can also affect cell behavior and the density of attachment ligands controls the amount of focal adhesions in a cell [8].

While all cells have usually direct access to glucose, amino acids, and other growth factors in 2D cultures, the availability of these nutrients depend on diffusion rates and local environments within the scaffold in 3D cultures [8]. Therefore, both 3D and 2D cell culture conditions affect to cells which have differential expression of genes involved in signal transduction as human epidermal growth factor receptor 2 (HER2) signaling, cellular movement, cell-to-cell signaling, cellular growth, and morphology [7,12-14].

Spheroid formation is one of the most well characterized models that mimic avascular tumor nodules or micro-metastases. Spheroids which are known as microtumors, they are aggregates of cells grown in suspension or embedded in a 3D matrix using 3D culture methods [4-13]. 3D spheroids can be used for assays that are drug screening, tumor growth and proliferation, immune interactions, invasion, matrix remodeling and angiogenesis [15].

There are four general methods of spheroid formation; suspension culture, nonadherent surface methods, hanging drop methods, and microfluidic methods. The hanging drop technique is one of the simplest and cheapest methods inside of them [5,10-15]. Although they have disadvantage as producing variable size spheroids, low throughput, hard to handle, long-term culture, they provide an efficient way to obtain biological insights that are often lost in 2D platforms [15].

For this reason, the 96-well hanging drop was chosen to form spheroids on breast cancer cell lines (JIMT, MCF-7, T-47D, BT-474). According to manufacturer cell concentrations were given 5×10^3 cells/well into Perfecta3D™ 96 well hanging drop plate, there are also some articles which include the seeding density, from as few as 50 cells to as many as 1.5×10^4 cells, allows production of varying spheroid sizes. One of the problem of 3D systems is that you cannot work with cells in

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excess. For this purpose, it was aimed to test if hanging drop is a useful method to form spheroids at density of 2.5×10^4, 5×10^4, 7.5×10^4, 10^5 cells/well or not, and forming spheroids in excess cells for the first time.

Materials and Methods

Sources of cell lines and material

Human breast cancer JIMT, MCF-7, BT-474 and T-47D cells were obtained from the American Type Culture Collection (ATCC), USA. All reagents used for cell culture, including Dulbecco's Modified Eagle's Medium (DMEM), Minimum Essential Medium (MEM), Roswell Park Memorial Institute (RPMI) 1640 medium were purchased from Biological Industries, USA. Fetal Bovine Serum (FBS), 0.25% trypsin-EDTA, 2 mM L-glutamine were purchased from Sigma, USA. Perfecta3D™ 96-Well Hanging Drop Plate was purchased from 3D BiomatrixTM.

Cell culture

JIMT and MCF-7 were grown respectively, in DMEM and MEM supplemented with 10% FBS. T-47D was grown in RPMI-1640 supplemented with 20% FBS and BT-474 was grown in RPMI-1640 supplemented with 20% FBS, 2 mM L-glutamine, 0.01 mg/ml human recombinant insulin.1% penicilin-streptomycin antibiotic (10,000 IU/mL and 10,000 µg/mL) were also added inside of media. The media was stored in a 4°C refrigerator and heated in a 37°C water bath prior to use. Cancer cell lines were recovered slowly from cryopreservation. The medium was changed every 2 days and cells were passaged weekly

Hanging drop plate method

Briefly, when cells reached confluent monolayer in a T-75 flask, washed twice with PBS (pH 7.4) and treated with 0.25% trypsin-EDTA, then resuspended in fresh medium. They were centrifuged (400 rpm for 10 minutes) and counted to calculate the volume needed. Cell density was estimated using a hemocytometer. According to manufacturer data, the device was made ready for assay. The device consists of 3 major parts: The lid, the hanging drop plate itself, and a tray on the bottom. According to calculation, cell suspensions of 40 µL (with concentrations of 2.5×10^4, 5×10^4, 7.5×10^4, 10^5 cells) were pipetted into each well located as part of the hanging drop plate piece in the center. 4 ml of distilled water was added into the peripheral water reservoir to keep the cells hydrated. The plate was sandwiched by a wellplate lid. The plate was labeled and was maintained at 37°C in humidified incubator with 5% CO_2 for five days to allow the spheroids to form. Cells were imaged daily to check for aggregation and cell proliferation. The growth media was exchanged every other day by taking 10 µL media from a drop and adding 14 µL fresh media into a drop to provide enough nutrients for cells and to prevent osmolality shift of the media. The fluorescence microscope examination and Image J software were used.

Results

According to our findings, most spheroids were more scattered in appearance and showed limited compactness and rounded shape after experimenting with different concentrations. Spheroid formations were determinated within 72 hours and they became a bit darker in color indicating more compactness. It was shown that the morphological appearance of 3D spheroid was cell line dependent (Figures 1 and 2). The fluorescence microscope examination revealed that the 96-well hanging drop plate is useful at density of 2.5×10^4, 5×10^4, 7.5×10^4, 10^5 cells/well that are more than cells from the protocol that is given from manufacturer. Although spheroids remained proliferative for five days, their pictures were taken only after spheroid were formed exactly.

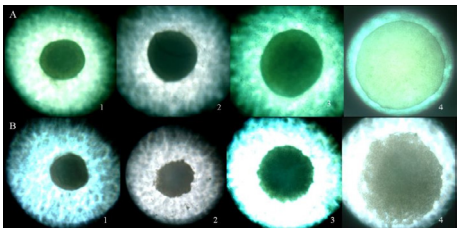

Figure 1: Spheroid formation occured within 72 hours and image of spheroids were taken by fluorescence microscope in the third day. A. Images of spheroids were belong to BT-474 cell at a density of 2.5×10^4, 5×10^4, 7.5×10^4, 10^5 (4x objective, number of the images respectively 1,2,3,4) B. Images of spheroids were belong to MCF-7 cell at a density of 2.5×10^4, 5×10^4, 7.5×10^4, 10^5 (4x objective, number of the images respectively 1,2,3,4) C. Images of spheroids were belong to T47D cell at a density of 2.5×10^4, 5×10^4, 7.5×10^4, 10^5 (4x objective, number of the images respectively 1,2,3,4). Images of spheroids were belong to JIMT cell at a density of 2.5×10^4, 5×10^4, 7.5×10^4, 10^5 (10x objective, the number of images respectively; 1,2,3,4).

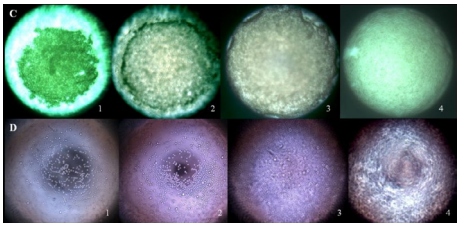

Figure 2: C. D. Images of spheroids were taken by fluorescence microscope (10x objective) at density of 10^5 in the third day. Images were belonging to BT-474, MCF-7, T-47D and JIMT cells (the number of images respectively; 1,2,3,4).

Discussion

Cancer cell spheroid formation is one of the most well characterized model that known as multicellular tumor spheroid [4,11]. 3D spheroids could be used for studies which include cell function in an avascular tumor microenvironment, drug therapies, tumor angiogenesis and tumor–immune cell interactions [15-17]. There are four general methods of spheroid formation and hanging drop was used in this study. The device consists of 3 major parts: the lid, the hanging drop plate itself, and a tray on the bottom We pipetted cell suspensions into each well located as part of the hanging drop plate piece in the center. Actually, concentrations were critical when plating hanging drops because if the spheroid is too heavy it could fall from the plate. PBS, water, or another buffer solution can be placed in the reservoir to keep the cells hydrated once the plate is placed in the tissue culture incubator. In this system, spheroids remain hydrated to keep cells viable. We exchanged 10 µL media and replace with 14 µL fresh media to provide enough nutrients for cells and to prevent osmolality shift of the media.

In this study, we used excess cells and it was shown that spheroid formation occurred within 72 hours. We observed most spheroids were more scattered in appearance after coated. The spheroids became a bit darker in color indicating more compactness and the building of cell layers creating the 3D spheroid structure. The fluorescence microscope examination revealed that the morphological appearance of 3D spheroid was cell line dependent.

Our aim was to test if hanging drop is an useful method to form spheroids at density of 2.5×10^4, 5×10^4, 7.5×10^4, 10^5 cells/well or not. Although hanging drop plate has disadvantages as hard to handle and long-term culture, we observed that spheroid remained proliferative for five days. Although pipetting should be careful while studying,

it is also very easy to transfer spheroids in this system. In a nutshell, hanging drop is a cheap and very practical method to form spheroids at these densities.

Conclusion

According to this study, more cells were used compare with the protocol that is given from manufacturer. The importance of our work is that spheroids were formed first time at high density.

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