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Mini Review Open Access

Technical Aspects of 3-Dimentional Culture of Mammalian Cells

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Abstract

Three dimensional-tissue culture systems of human solid tumor cells has been implemented for effective chemotherapeutic screening and to avoid the disadvantages of the over simplified monolayer tissue culture system. The influence of tumor microenvironment to tumor therapeutics and screening of new anticancer drugs has been found to significant. The complex tumor microenvironment is poorly represented in the simple monolayer tissue culture system. The 3D culture models closely imitate several in vivo conditions of solid tumors in terms of geometry and expression of microenvironment. . However, several technical obstacles might discourage the use of this culture model. In the current mini-review, we are presenting the exact protocol for culturing mammalian cell as three dimensional tissue culture systems (multicellular spheroids and multicellular layers) and how to utilize these models for anti-cancer assessment.

Introduction

Cancer problem: relation to screening platform

Currently, cancer is the leading cause of death worldwide regardless of the discovery of several dozens of novel anticancer drugs [1]. Despite the oversimplified monolayer culture system, it serves as a common tool for anti-cancer screening; however, it lacks clinical relevance to solid tumors *in-situ*. Although national cancer institute cell line panel (NCI-60 cell line) test has been used for anti-cancer drug screening, it presents limitations due to oversimplified two dimensional culture model [2]. Three dimensional-tissue culture of human cells has been introduced for effective anti-solid tumor drug screening [3]. The 3D culture model simulates many of the in vivo microenvironment conditions of solid tumors such as, lack of vasculature, abundance of extracellular matrix (ECM), and cell-cell communication [4-7]. Different types of 3D culture models have been developed for the anti-cancer drug screening [3,8-10]. Herein, we will present the exact protocol for culturing mammalian cell as three dimensional tissue culture systems (multicellular spheroids and multicellular layers) and how to utilize these models for anti-cancer assessment in terms of pharmacodynamics and pharmacokinetics.

Materials and Methods

Preparation of multicellular spheroid (MCS) 3-D tissue culture of tumor cells by liquid overlay technique

Plate preparation:

- Prepare 0.75-1.5% agarose solution by adding sea plaque agarose powder to serum free media under sterile condition (laminar flow cabinet), incubate at 60°C water bath for 30 min.
- Confirm dissolution by incubation in microwave oven for 30 seconds 2-3 times.
- After complete dissolution of agarose, add 60 µl of the prepared solution to each well of the U-shape 96-well plate. N.B. Previous step must be carried out rapidly (before congealing) and accurately (without any air bubbles); it is recommended to use pre-warmed (60°C) pipette tips
- Wait the agarose to congeal in the 96-well plates and keep the plates open in the laminar flow cabinet over night under UV-light to confirm sterility.
- Coated plates could be stored at 4°C for up to 4 weeks.

Seeding

- Detach cells (Using trypsin-EDTA or alternatives) from monolayer culture according to lab routine protocol.
- Collect detached cells in 10% FBS containing media and centrifuge (200 Xg) for 10 min at 4°C.
- Discard supernatant media and re-suspend cells in 10% FBS containing media, count viable cells using trypan blue exclusion routine protocol.
- Adjust cell number per 100 µl using 10% FBS containing media talking in consideration the doubling time of the cell line in use.

N.B. recommended seeding density might be 10^4 cells/100 µl.

- Inoculate exactly 100 µl of the prepared cell suspension into the U-shape bottom, agarose coated 96-well plate.
- After seeding in agarose-coated plates, incubate without shaking in CO_2 incubator for 24 h.

N.B. pre-shaking period might be different according to the aggregation ability of the cell line in use; cells with more aggregation (i.e. DLD-1 and HT-29) ability might require at short as 12 h while other cells might require periods up to 24 h.

- Start the 96-well plate orbital shaker with 15 min periodic On/Off shaking cycles at 450 rpm for up to 7 days in conventional CO_2 incubator.

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Media change for MCS:

The high cellular content relative to the media volume used in the liquid overlay technique mandates media change every 48 h during MCS culture procedures since the start day of orbital shaking.

- Add extra 100 µl fresh media to each well of the MCS-containing 96-well plate.
- Gently, tilt the plate by 45° and slowly remove 100 µl media which results in 50% media change.

Drug treatment in MCS 3-D culture module:

- When MCS reach required size (300-500 µm diameter), transfer the appropriate number MCS's to corresponding wells of uncoated flat bottom 96-well plate (treatment module).

N.B. To facilitate the MCS transfer process, cut the distal part of 1000 µl pipette tip using sterile scissor to widen its opening.

- Remove as much as possible of the media transferred with the MCS's using narrow pipette tip (recommended 100 µl yellow tip) by tilting the plate and angle suction.
- Add 100 µl drug containing media to corresponding MCS's containing wells and incubate for the designed period
- Several assays are applicable to MCS after drug treatment such as, MTS assay, cell sprouting assay, dimension shrinkage assay, BrdU assay…etc

As shown in Figure 1, MCS grows steadily over a period of 11 days, Figures 1A and 1B with homogenous smooth surface as appeared by scanning electron microscopy (Figure 1C) and tissue like structure Figure 1-E with characteristic cell-cell communication appears in transmission electron microscopy image as tight junction and desmosomes (Figure 1D). Fully compact MCS can be easily transferred for treatment plate and tested for anticancer susceptibility. Other cell lines (A431, PCI-1, DLD-1 and SNU-484) can form similar characteristic MCS culture; however, some cell lines such as, A549, PC-14, PCI-13, PCI-50, AGS, SNU-216 and SNU-601 hardly result in loose aggregate that is not technically acceptable for anti-cancer drug assessment.

Preparation of multicellular layers (MCL) 3-D tissue culture of tumor cells

Transwell preparation (coating with collagen)

- Prepare 60% Ethyl alcohol, and 0.1 M Acetic acid and sterilize by filtration through 0.22 µm bacterial filter.
- Expose calf skin collagen type-III to UV-light for 30 min and then aseptically (under laminar flow cabinet) dissolve in 0.1 M Acetic acid by stirring for 3-5 h at 4°C to get 2.5 mg/ml stalk solution
- Aseptically dilute the collagen stalk solution (2.5 mg/ml) by 60% Ethyl alcohol to a final working solution of collagen (660 µg/ml)
- Spread 50 µl of the final collagen working solution on the top of the transwell membrane (i.e. Costar-3413®, pore size 0.4 µm; diameter 6.5 mm) and incubate over night under UV-light in the laminar flow cabinet.

Seeding:

- Insert the collagen-coated transwell into the 6/12 well culture module (Figure 2A).
- Detach cells (Using trypsin-EDTA or alternatives) from monolayer culture according to lab routine protocol.
- Collect detached cells in 10% FBS containing media and centrifuge (200 X g) for 10 min at 4°C
- Discard supernatant media and re-suspend cells in 10% FBS containing media, count viable cells using trypan blue exclusion routine protocol.
- Adjust cell number per 100 µl using 10% FBS containing media talking in consideration the doubling time of the cell line in use.

N.B. recommended seeding density might be 3x105 cells/100 µl.

- Inoculate exactly 100 µl of the prepared cell suspension into each collagen-coated transwell and incubate at CO_2 incubator for 4-6 h.
- Add another 100 µl of 10% FBS containing media onto the top of the seeded cells (slowly not to disturb the cells) and Incubate in CO_2 incubator for another 4-6 h.
- Add another (third) 100 µl of 10% FBS containing media to the internal chamber of the transwell insert, and add 75/150 ml of 10% FBS containing media to the external chamber of the 6/12 well culture chamber module to touch the lower transwell meniscus, and incubate over night in CO_2 incubator.
- Add another 50/100 ml of 10% FBS containing media to the external chamber of the 6/12 well culture jar until complete coverage of the transwell insert, and incubate in CO_2 incubator for 24 hrs without stirring.
- Start very slow magnetic stirring (60 rpm) for the total culture duration (5-15 days).

N.B. Only the media of the external chamber might be changed every 3-5 days according to the pH condition.

Drug treatment in MCL culture module

- For drug treatment, MCL must be transferred to treatment culture module (6-well plate with hanging adaptor) after careful, and complete old media decantation (Figure 2B).
- Add drug containing media to the top transwell chamber and drug free media (10% FBS) to the bottom chamber of the treatment module to touch the lower transwell meniscus (about 7 ml).
- Incubate according to your experimental design and several assays (mainly pharmacokinetic measurements) can be performed on MCL module such as, temporal drug penetration rate (from top to bottom chamber), tumor tissue drug distribution profile, immune histo chemical assessments, spatial viability … etc.

Several cell lines such as, DLD-1, HT-29, HT-1376 and J-2 can effectively constitute typical tissue like structure in the form of MCL after relatively short time (less than 7 days) (Figure 3A). also cell lines such as A431 can form similar characteristics MCL however after quite longer culture duration (up to two weeks) (Figure 3A). On the other

hand, cell lines like SiHa, SNU-484, SNU-601, PCI-1, and HeLa form either irregular friable MCL structure or lack the ability of forming MCL at all even after culture up to 15 days (Figure 3B).

Histological confirmation for 3-D culture formation For MCL

- Gently remove the media from the top chamber of the transwell insert
- Wash with ice-cold PBS solution by dipping in 2 ml containing 24-well plate
- Add 200 µl (OCT compound : 20 % sucrose in PBS; 1:2 v/v) and freeze immediately at -20°C
- Perform routine frozen section and H&E staining.

For MCS

- Collect 5-6 MCS's in one tube carefully.
- Remove the remaining media and wash with 1 ml cold PBS solution.
- Add 1 ml (OCT compound : 20 % sucrose in PBS; 1:2 v/v) and freeze immediately at -20°C
- Perform routine frozen section and H&E staining.

Results and Discussion

Applications of 3-D culture modules in drug discovery and anticancer screening

The effect of cytotoxic drug is markedly weaker against the MCS module of any cell line compared to the conventional monolayer screening culture module [11]. Yet, MCS system can be used effectively and more realistically for screening anti-cancer potential of new compounds [12]. Super complicated mathematical modeling is implicated in understanding the complex penetration problem of anti-cancer drugs within solid tumor microenvironment [10].

MCL culture was firstly introduced by Wilson and colleagues [13]; and by Tannock and colleagues [14] to measure the penetration rate and characteristics of chemotherapeutic transverse within solid tumor avascular layers . Despite secondary efficacy parameters, the main purpose of MCL culture module was intratumoral pharmacokinetics assessment [15]. We recently, utilized the MCL system for simultaneous efficacy (pharmacodynamics) and penetration (pharmacokinetics) portfolio spatial assessment within solid tumor avascular microenvironment for simple (paclitaxel) [11] and for genetic silencing designed drug (siRNA) [16].

In the current report, we have presented the technical aspects for generating example of 3-D tissue culture system and insights for its possible application in anticancer drug discovery and development field.

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