Application of hanging drop technique for stem cell differentiation and cytotoxicity studies

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Abstract The aim of our study is to explore the possibility of using an ancient method of culture technique- the hanging drop technique for stem cell differentiation and cytotoxicity testing. We demonstrate here a variety of novel applications of this age old technique not only to harness the differentiation potential of stem cells into specific lineages but also for cytotoxicity studies. Here we have prepared hanging drop cultures by placing 20 µl micro-drops of nutrient media and 10% Fetal Calf Serum (FCS) containing cells of interest on the lids of 60 mm dishes. Bottom plates of the dishes were filled with sterile Phosphate Buffer Saline (PBS) to avoid desiccation of samples. Lids were then placed on the bottom plates to achieve hanging drop cultures. We utilized this technique for cultivation of ciliated epithelia to study cytotoxicity and differentiation of bone marrow stromal cells. Most importantly the modified culture technique presented here is simple, economical and cost effective in terms of the time taken and the reagents required and are amenable to goal specific modification such as cytotoxicity testing. It is advantageous over the existing system in terms of retention of viability and functionality for longer duration and for providing three dimensional

growth micro-environment making it useful for organotypic cultures and *in vivo* simulation.

Keywords Extra-cellular matrix · Microenvironment · Organ culture · Three dimensional culture · Tissue engineering · Stem cells

Introduction

Historically hanging drop technique has been used in animal tissue culture since its inception, to generate primary cultures of fibroblasts from chick heart explants using solid substrata such as plasma clot (Harrison 1907; Carrel 1992). The hanging drop technique using plasma clot is advantageous as it not only provides an excellent *in vitro* system to study developmental regulation during embryogenesis but also offers a three dimensional micro-environmental niche to the developing tissue. However, the importance of this technique for three dimensional cultures was not realized as the major emphasis was on proliferation of the cells into two dimensions representing classical tissue culture. The present day tissue culture has seen the technological advances on all walks of *in vitro* life and more so for achieving three dimensional growth for tissue engineering applications. In order to obtain organotypic growth so as to avail cell to cell and cell to matrix interaction, it is desirable to have three dimensional structures. In a

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three dimensional environment, cells are expected to develop tissue like higher order intercellular structures that orchestrate cellular functions including proliferation, differentiation, apoptosis, and angiogenesis (Kelm et al. 2004). In addition to its use for tissue explant cultures to initiate primary cell cultures, the hanging drop technique is being widely used for several *in vitro* studies. Kawakami et al. has examined intra-thymic T-cell development by employing the same culture technique (Kawakami et al. 1999). The technique has been well documented with embryonic stem cells to generate embryoid bodies (Ali et al. 2004). In 1981, Medvinskii et al. proposed the modification of “hanging drop” method. This modified method of “suspension drop” permits availability of the nutrient medium at the expense of capillary forces (Medvinskii and Kolesnichenko 1981).

We demonstrate here for the first time the suitability of the hanging drop technique to harness the differentiation potential of bone marrow stem cells into desired cell lineages and cytotoxicity testing especially for volatile substances.

Materials and methods

Cultivation of mouse bone marrow stromal cells

Mice (Balb/c) femur bones were collected aseptically. Bones were then flushed with medium to get single cells of bone marrow as described earlier (Banerjee et al. 2005). After washing, cells were seeded with Iscove's Modified Dulbecco's Medium (IMDM, Gibco) containing 20% FCS (Gibco). The floating population was removed after 72 h of incubation. Adherent cell population was maintained further in IMDM containing 10% FCS. Adherent cells were passaged for two generations to get pure and homogenous stromal cell population.

Organoid body formation in hanging drop

Drops of 20 μ l containing 500–700 bone marrow stromal cells suspended in IMDM with 10% FCS were placed on the inner side of the lid of 60 mm tissue culture dishes (Falcon). Dishes were then filled with PBS to avoid loss of nutrients through evaporation and incubated for 48 h in hanging drop at 37°C in CO₂ incubator.

Differentiation of bone marrow organoid bodies into desired cell lineages

Organoid bodies of bone marrow stromal cells formed in hanging drop after 48 h of incubation were re-seeded into culture plates containing IMDM with 10% FCS. Confluent cell monolayers thus formed were stimulated with lineage specific differentiation medium viz; adipogenic medium—1 μ M Dexamethasone (Sigma), 0.5 mM isobutyl methylxanthine (Sigma), 100 mM indomethacin (Sigma) and 10 μ g ml⁻¹ insulin (Sigma), chondrogenic medium—1 $\times$ insulin-transferrin selenium (Gibco), 1 mM sodium pyruvate (Gibco), 0.1 μ M dexamethasone, 397 μ g ml⁻¹ ascorbate-2-phosphate and 10 ng ml⁻¹ transforming growth factor (R & D Systems, Minneapolis, MN, USA) and osteogenic medium—0.1 μ M Dexamethasone, 10 mM β -glycerophosphate and 100 μ M ascorbate-2-phosphate to get desired cell differentiation. Adipogenic differentiation was evaluated by the cellular accumulation of neutral lipid vacuoles that were stained with Oil-red ‘O’ (Sigma). Osteogenic differentiation was confirmed by the expression of alkaline phosphatase staining and chondrogenic differentiation was detected by safranin orange (Sigma) staining.

Preparation and cytotoxicity testing of tracheal organ cultures in hanging drop

Newborn chick tracheal rings were prepared as described earlier (Bhonde et al. 1983). Instead of putting tracheal rings in tissue culture plates, we placed them in hanging drop consisting of Minimum Essential Medium with 10% fetal calf serum. Control sets consisted of PBS in plates where as experimental sets consisted of a variety of volatile substances such as ethanol, methanol, camphor and benzene. Plates were then incubated at 37°C for 72 h.

Results and discussion

Effect of hanging drop culture technique on differentiation potential of bone marrow stromal cells

We found that bone marrow stromal cells aggregated and formed dense cell masses called organoid

bodies (Fig. 1a). These ‘organoid bodies’ upon seeding in culture plates, proliferated to confluency indicating retention of their self-duplication capacity (Fig. 1b). Finally upon stimulation with specific differentiation medium these cells differentiated into adipocyte (Fig. 1c), osteocyte (Fig. 1d) and chondrocyte (Fig. 1e) lineages. We also observed that if the stromal cells were allowed to form dense cell masses in hanging drop with lineage specific differentiation medium, the process was enhanced and organoid bodies thus obtained, demonstrated lineage specific differentiated cells (data not shown). This feature indicates that hanging drop technique can be applied to study the impact of the medium composition on tissue specific differentiation pattern. This could be the result of intimate cell to cell contact and appropriate exchange of autocrine/paracrine factors permitted by the micro drop niche consisting of growth/differentiation factors. Our study clearly demonstrates the use of this technique to exhibit amphoteric behavior of the bone marrow stem cells i.e. to serve dual purpose of maintenance of undifferentiated state while retaining its differentiation potential to a desired lineage.

We have further carried out the quantitative analysis of the differentiated progenies present when the

mesenchymal cells were allowed to undergo differentiation in two dimensional culture conditions i.e.; conventional culture systems and under three dimensional cultures i.e.; hanging drop. We counted the adipocytes, osteocytes and chondrocytes based on the positive expression towards oil red O, alkaline phosphatase and safranin O in both set-ups. The data presented in Table 1 do not exhibit any difference in the percent positive differentiated cells in any of the differentiation lineage. Thus it is clear that three dimensional culture system viz, hanging drop neither have positive influence nor negative influence on the differentiation potential of bone marrow mesenchymal stem cells; however it permits long term survival and maintenance of stemness of bone marrow which is prerequisite for stem cell research.

Suitability of hanging drop technique for cytotoxicity studies

We propose here yet another novel application of the said method for testing toxic effect of volatile compounds which otherwise are difficult to test on cell/organ cultures. We presumed that exposure of tracheal rings in hanging drop (as shown in Fig. 2) to volatile substances producing vapors will directly affect ciliary activity. We found that all above agents

Fig. 1 Bone marrow stromal cells in hanging drop. Organoid bodies of bone marrow stromal cells formed in hanging drop were re-seeded in tissue culture dishes (a), organoid bodies formed confluent stromal cell monolayer (b) stromal cells upon stimulation with specific differentiation medium gave rise to cells of adipogenic (c), chondrogenic (d) and osteogenic lineages (e)

Table 1 Table represents the quantitative data of percent positive differentiated cell progenies viz, adipocytes, osteocytes and chondrocytes in two dimensional as well as three dimensional culture set-ups. Values are the mean $\pm$ SEM

Culture condition	Adipocytes (% differentiated progenies)	Osteocytes (% differentiated progenies)	Chondrocytes (% differentiated progenies)
Two dimensional (conventional)	73.0 $\pm$ 3.79	78.33 $\pm$ 2.33	48.67 $\pm$ 2.03
Three dimensional (hanging drop)	70.0 $\pm$ 4.62	83.33 $\pm$ 1.56	43.27 $\pm$ 3.24

Fig. 2 Cytotoxicity of volatile substances on tracheal organ cultures in hanging drop. Newborn chick tracheal rings were cut under sterile conditions and placed in (a) hanging drop, (b) a single tracheal ring in hanging drop

exerted their toxic effect on tracheal rings grown in hanging drops as evidenced by disturbed ciliary activity within 5 h and distorted morphology with accumulation of mucus in lumen of tracheal rings within 24 h (Table 2) where as the control tracheal rings in hanging drop remained viable as well as functional up to 20 days in the drop. In any culture system the cells are submerged in nutrient medium and hence it is difficult to assess the effect of volatile substances on cell physiology. Therefore it is

advantageous to use hanging drop for this purpose simulating near *in vivo* situation.

Hanging drop technique has its own inherent merits, which makes it an attractive model depicting *in vivo* conditions. Considering the *in vivo* scenario, one realizes that each organ constitutes the functional cell-mass within extra cellular matrices in a three dimensional configuration. Researchers have used natural/artificial matrices to design three dimensional microenvironment for maintenance of tissue architecture *in vitro* viz collagen I matrix to generate skin organotypic cultures (Gron et al. 1999), porous titanium fiber mesh to study osteoblast differentiation (Sikavitsas et al. 2003), Calcium cross linked alginate to study marrow cell proliferation (Wang et al. 2003) and porous polyvinyl formal (PVF) resin scaffold to study *ex vivo* expansion of human progenitor cells (Tun et al. 2002). All these data confirm the superiority of three-dimensional growth conditions over two dimensional growth. In a recent study by Li et al. (2005), the authors have demonstrated that hanging drop culture facilitate the process of differentiation of immature thymocytes into mature T cells by providing direct interaction among cell population *in vitro*. Gutierrez et al. (Gutierrez et al. 2005) have compared the hanging drop cultures to that of 'methylcellulose cultures' to study the differentiation

Table 2 Table represents the effect of 5 h and 24 h exposure of toxic vapors of various substances on several properties of newborn chick tracheal rings, placed in hanging drop cultures

S. no.	Parameters	Exposure time (5 h)	Exposure time (24 h)
1.	Ciliary activity	Inhibition	No activity seen
2.	Mucus accumulation in the lumen of the rings	Mucus started accumulating	Lumen of the rings were filled with mucus
3.	Distorted cell morphology	Ring morphology was intact	Tracheal rings lost their structure completely

§ Volatile substances used in the study were ethanol, methanol, camphor and benzene

§ Tracheal rings placed in control medium i.e.; MEM (Minimum Essential Medium) + FCS (10%) remained viable as well as functional up to 20 days in the hanging drop

of primary erythroid progenitors. Their data demonstrated that the established methods for *ex vivo* culture of primary erythroid progenitors, such as methylcellulose-based burst-forming unit-erythroid (BFU-E) and colony-forming unit-erythroid (CFU-E) assays, allow the detection of committed erythroid progenitors but are of limited value to study terminal erythroid differentiation. In this context hanging drop culture is a practical alternative that enables a comprehensive assessment of the behavior of primary erythroid cells *ex vivo* in the context of genetic and drug-induced perturbations. The use of three-dimensional *in vitro* culture systems has also been studied to reconstruct some of the cellular microenvironments such as in brain tumor invasion (Duca et al. 2004). It has been highlighted in the study that the spheroids prepared by ‘spinner culture’ showed histological evidence of central necrosis, whereas the use of hanging drops broadens the applicability of three-dimensional *in vitro* assays examining brain tumor invasiveness. Hanging drop cultures thus represents an attractive alternative for maintenance of a broad range of cell lines with 100% efficiency (Kelm et al. 2003). The method has applications for basic studies of physiology and metabolism, tumor biology, toxicology, cellular organization, and the development of bioartificial tissue.

Our present study employing hanging drop indicates that the technique although of historical significance can be gainfully utilized for variety of applications including stem cells biology, organ culture and cytotoxicity studies. On this background the present data demonstrate the application of this technique for three dimensional growth of cells. It is simple, economical and can be adapted to accommodate any *in vitro* culture system, thus making it a valuable tool for developmental biology, toxicology as well as tissue engineering.

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