OSUCCC Leukemia Tissue Bank Protocol: Primary Cell culture of human bone marrow aspirate: Culture and Harvest of Fibroblasts

OSUCCC Leukemia Tissue Bank Laboratories Procedure: Establishment of Primary Cell culture of human bone marrow aspirate: Culture and Harvest of Fibroblasts Cells			Effective: 8-20-2013
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Procedure No.	Supersedes:	Created/Review Date:	Number of Pages:
Version 1.0		2/2/2014	4
		11/24/2014	4
		11/30/2015	4

1.0 PRINCIPLE

Bone marrow stromal cells (BMSCs, frequently also called MSCs) represent a cell population within the bone marrow, a subset of which contains multipotent stem cells. Their primary role is to produce and maintain both bone tissue and bone marrow microenviron- ment necessary for hematopoiesis (Friedenstein et al., 1966, 1968). The latter is achieved by secreting a wide variety of different cytokines and growth factors, many of which also have a regulatory role in immune processes. BMSCs have recently been introduced into the field of immunobiology after their successful clinical use in GVHD was reported in 2004 (Le Blanc et al., 2004). Since then numerous studies have confirmed and expanded the knowledge on the immunosuppressive potential of BMSCs in various in vitro and in vivo models (Uccelli et al., 2008). Although the immunomodulatory capacity of BMSCs is well established, there are still many unanswered questions regarding the cytokines, chemokines, receptors, and molecular pathways that play a role in this effect.

Cell culture is a method in which cells are grown under controlled conditions, generally in a favorable artificial environment. This technique is used to increase specific cell lines for a variety of research purposes. Cell culture is one of the major tools used in cellular and molecular biology, providing excellent model systems for studying the normal physiology and biochemistry of cells, the effects of drugs and toxic compounds on the cells, and mutagenesis and carcinogenesis. It is also used in drug screening and development, and large scale manufacturing of biological compounds. The major advantage of using cell culture for any of these applications is the consistency and reproducibility of results that can be obtained from using a batch of clonal cells[1]. Fibroblasts are one of the cell types that can be grown in artificial conditions. They play a role in maintaining the structural integrity of connective tissues[2] and can also be used to derive stem cells for research. Thus, fibroblasts are essential for many research studies.

2.0 SPECIMEN

Bone marrow (BM) aspirate in EDTA lavender top tube. Peripheral blood is used if the BM aspirate is a dry tap.

3.0 MATERIALS AND REAGENTS

Dulbecco's Modified Eagle Medium (DMEM) + 20% Fetal Bovine serum (FBS) + 1% penicillin/streptomycin glutamine (Life Technologies #11965-092, #16140-071, 10378-016)

DMEM freezing media; 45% DMEM, 45% FBS (Life Technologies #16140-071), 10% DMSO (Sigma Aldrich #D2650-100ml)

TrypLE (Trypsin) Solution (Life Technologies # A12177-02)

Dulbecco's Phosphate Buffer Saline (D-PBS, Life Technologies #19140-144)

Trypan blue (Life Technologies #15250-061)
15ml conical tubes
Eppendorf (microcentrifuge) tubes, 1.2ml
Sterile pipet tips (200µl and 1000 µl)
Micropipets (20-200µl and 1000 µl)
Pipet Aid
CO_2 Incubator (37°C)
Sterile Cell scrapers
Sterile tissue culture flask (T-25)
Sterile cell culture dish
Hemacytometer – Brightline
70% isopropyl alcohol
Wexcide solution
Controlled rate freezing container

4.0 EQUIPMENT

Biological safety cabinet, with vacuum aspirator set up
Benchtop centrifuge with swinging bucket rotors to hold 15 and 50cc conical tubes, with adaptor covers.
Microcentrifuge
Standard light microscope (4x and 10x objectives)
Biological Safety Cabinet
Inverted light microscope (4x, 10x and 40x objectives)
CO_2 Incubator – (37°C, 5% CO_2)

5.0 QUALITY CONTROL AND SAFETY

It is recommended that specimen collection be carried out in accordance with NCCLS document M29-T2. No known test sample can offer complete assurance that human blood samples will not transmit infection. Therefore, all derivatives are potentially infectious. Decontaminate biological safety cabinet surface with Wexcide solution. Always spray alcohol on the caps before opening solutions. The alcohol can be dried off using gauze. Disposable latex or nitrile gloves are worn at all times when handling samples, packages, and sample documentation. Dispose of gloves as soon as work is completed. Wash hands as soon as gloves are removed. Observe "NO GLOVE" areas. Pipets or pipet tips should never touch sides (inside or outside) of any bottles. Change pipet or tip if you think this has occurred. All materials are discarded in biohazard boxes. Broken tubes should be wrapped in a layer of bench cover, absorbent material, taped and discarded with biohazard waste.

6.0 PROCEDURE

6.1 Establishment of primary culture

6.1.1 In biosafety cabinet, sterilize workspace and equipment with Wexcide solution. Warm complete DMEM in 37°C water bath for at least 10 minutes.

6.1.2 Label a sterile T-25 tissue culture flask with unique sample ID, date, and users initials on the side of a sterileT-25 flask.

6.1.3 When complete DMEM media is ready (warm to touch), aliquot 5 mL of the solution into a sterile 15 mL conical tube.

6.1.4 Aliquot 150 uL of patient BM aspirate into the sterile 15 mL media tube and mix by inverting the tube carefully.

6.1.5 Carefully, without touching the side, transfer the mixed media and aspirate to a T-25 flask with a pipette gun.

6.1.6 Incubate sample at 37°C, 5% CO_2. Observe culture, making notes on observations and change the media every 5-7 days by removing 2 mL of and replacing it with 2 mL of fresh

complete DMEM.

6.1.7 Repeat step 6.1.6 until culture achieves 80-85% confluence.

6.2 Harvest of cultures

6.2.1 Method 1 – Sterile Cell Scraper

6.2.1.1 Working in biosafety cabinet, decontaminate workspace and equipment with Wexcide solution. Warm complete DMEM in 37°C water bath for at least 10 minutes.

6.2.1.2 In the hood, scrape the side of T-25 flask gently with a sterile cell scraper. With a pipet aid, rinse the side of the flask with its own media before transfer it to a 15 mL conical.

6.2.1.3 Add 2-3 mL of modified DMEM to T-25 flask and check for any remaining cells in the flask with inverted microscope. Repeat step 6.2.1.2 if necessary.

6.2.1.4 Centrifuge the sample at 1100 RPM for 10 minutes. Remove supernatant by aspiration being careful not to disturb the pellet.

6.2.1.5 Depending on the size of the pellet, re-suspend the sample with complete DMEM (200 uL-1000 uL) and transfer to a sterile Eppendorf tube.

6.2.1.6 Perform cell count by preparing a 1:2, 1:5, or 1:10, cells to trypan blue dilution. Then aliquot 10 uL of the diluted sample into each of the two chambers on the hemacytometer and observe under the microscope.

6.2.1.7 After the cell count is determined, spin the sample at 1100 RPM for 10 minutes. Remove supernatant by aspiration.

6.2.1.8 Re-suspend the sample in 1 mL DMEM freezing media and store the sample in -80°C freezer overnight in a controlled-rate freezing container before transferring it to a liquid nitrogen freezer for long-term storage.

6.2.2 Method 2 – Trypsinization with TrypLE solution

6.2.2.1 Working in biosafety cabinet, sterilize workspace and equipment with 70% ethanol. Incubate complete DMEM, D-PBS, and TrypLE in 37°C water bath for at least 10 minutes. Aspirate spent medium from culture and discard.

6.2.2.2 Wash monolayer with 5 mL of warmed D-PBS. Aspirate D-PBS and discard.

6.2.2.3 Add 1-2 mL of TrypLE to flask. Ensure complete coverage of cell monolayer with TrypLE.

6.2.2.4 Incubate at 37°C until cells have detached (about 2 minutes). Observe cell monolayer using an inverted microscope to ensure complete cell detachment from the surface of the flask. Gently tap flask to dislodge cells if necessary.

6.2.2.5 Add 5-10 mL of pre-warmed DMEM to flask. Tilt the flask in all directions to thoroughly rinse flask.

6.2.2.6 Transfer cell suspension to a 15mL conical tube and centrifuge at 100g for 10 minutes.

5.2.2.7 Discard supernatant and re-suspend the sample in 1 mL DMEM freezing media and store the sample in -80°C freezer overnight in a controlled-rate freezing container before transferring it to a liquid nitrogen freezer for long-term storage.

7.0 LIMITATIONS OF THE PROCEDURE

The temperature of the DMEM can affect cell growth. The bottle should always be pre-warmed to 37°C before used for cell culture. Lack of nutrients and contaminations may lead to low or no fibroblast growth. It is important to regularly monitor the growth. Fibroblasts usually divide only a limited number of times before losing their ability to proliferate. Thus, harvesting should be done in a timely manner.

8.0 REFERENCES

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