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Research Article

ISOLATION OF MESENCHYMAL-LIKE CELLS FROM WHARTON'S JELLY OF UMBILICAL CORD

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ABSTRACT

The umbilical cord[1] is a noncontroversial source of mesenchymal-like stem cells[2-6] Mesenchymal-like cells are found in several tissue compartments of the umbilical cord, placenta, and decidua. Here, we confine ourselves to discussing mesenchymal like cells derived from Wharton's Jelly, called umbilical cord matrix stem cells (UCMSCs). Work from several laboratories[3].Shows that these cells have therapeutic potential, possibly as a substitute cell for bone marrow-derived mesenchymal stem cells for cellular therapy. There have been no head-to-head comparisons between mesenchymal cells derived from different sources for therapy; therefore, their relative utility is not understood. In this chapter, the isolation protocols of the Wharton's Jelly-derived mesenchymal cells are provided as are protocols for their in vitro culturing and storage. The cell culture methods provided will enable basic scientific research on the UCMSCs. Our vision is that both umbilical cord blood and UCMSCs will be commercially collected and stored in the future for preclinical work, public and private banking services, etc. While umbilical cord blood ban standard operating procedures exist, the scenario mentioned above requires clinical-grade UCMSCs. The hurdles that have been identified for the generation of clinical-grade umbilical cord-derived mesenchymal cells are discussed.

Key words: Mesenchymal-Like Cells, Wharton's Jelly, stem cells.

INTRODUCTION

Mesenchymal stromal cells (MSCs), as defined by the International Society for Cellular Therapy, are plastic-adherent cells with a specific surface phenotype that have the capacity to self-renew and to differentiate into various lineages including bone, cartilage, and adipose .Such cells can be derived from several different sources, such as trabecular bone, adipose tissue, synovium, skeletal muscle, dermis, pericytes, blood, and bone marrow. MSCs derived from bone marrow and adipose tissue have been studied extensively[8-11]. MSCs derived from bone marrow can be differentiated into bone, cartilage, tendon, muscle, adipose tissue, and hematopoietic cell-supporting stroma. Thus, they are candidates to treat patients suffering from bone disorders, heart failure, etc. Since MSCs can be isolated from adults in significant number, they have been examined closely for therapeutic utility. For example, MSCs support the ex vivo expansion of hematopoietic stem cells , act as immune modulators, release cytokines and growth factors, and they home to sites of pathology. It is estimated that more than 50 clinical trials are ongoing using bone

marrow-derived MSCs for a variety of indications, for example, acute myocardial infarction[18-20], stroke, and graft versus host disease. Nevertheless, there are limitations associated with MSCs derived from bone marrow for cell-based therapy. For example, collection of MSCs from bone marrow is an invasive and painful procedure. In normal aging, the marrow cavity fills with yellow fat. Thus, there may be difficulty in obtaining MSCs from older individuals. Along these lines, differences have been found between bone marrow-derived MSCs collected from the fetus versus adult-derived MSCs. For example, fetal MSCs have a longer life in vitro compared to adult-derived MSCs (MSCs derived from adults have a useful lifespan in vitro of about five passages In addition to bone marrow, MSCs may be derived from adipose tissue. While adipose-derived MSCs (ASCs) have been studied less than bone marrow-derived MSCs, ASCs may be induced to differentiate into osteocytes), cartilage, and cardio myocytes[15-16.] and they display both similar surface phenotype and immune properties to bone marrow-derived MSCs .While there is no shortage of the adipose material within the United States, the procurement of adipose tissue involves an invasive and painful surgical procedure. There is no comparison work done to evaluate ASCs from the fetus with adult-derived ASCs demonstrated that cells derived from the Wharton's Jelly in umbilical cords (so called umbilical cord matrix cells or UCMSCs) have properties of MSCs. While UCMSCs have surface phenotype differentiation capability and immune properties similar to MSCs derived from bone marrow and adipose. UCMSCs are more similar to fetal MSCs in terms of their in vitro expansion potential. In contrast to bone marrow- and adipose-derived MSCs, UCMSCs are isolated from the umbilical cord following birth and may be collected following either normal vaginal delivery or cesarean section. As described below, UCMSCs are easily expandable in vitro, and may be cryogenically stored, thawed, and reanimated. While the collection process for human materials is elaborated on here, UCMSCs have also been isolated using modified protocols from dog, cat, rat, mouse, horse, bovine, and swine umbilical cord .Human UCMSCs grow as plastic-adherent cells, express a surface phenotype similar to other MSCs and differentiate into multiple lineages Umbilical cord matrix cells have been safely transplanted and ameliorated symptoms in an animal model of Parkinson's disease , neural damage associated with cardiac arrest/resuscitation retinal disease and cerebral global ischemia[22] Finally, UCMSCs that have been mitotically inactivated can be used as a feeder layer for embryonic stem cells. Because of their physiological properties, and because of the ease of isolation, expansion, and banking capability, UCMSCs may be useful clinically and experimentally. These detailed protocols should enable further research on this interesting population.

Methodology

Isolation of Cells

Use of umbilical cord tissue from human subjects requires Institutional Review Board (IRB) approval and a signed informed consent form. However, since DNA testing makes UCMSCs individually identifiable, an IRB may assign a protocol number and track the work. The informed consent outlines your project, and must be signed by the donor, and witnessed. Cords are specifically excluded from individuals with questionable health status, for example, stillbirth, preeclampsia, infectious disease, STD, or hepatitis-positive mother. After the delivery of the baby, the umbilical cord is collected and stored in a sterile specimen cup containing 0.9% normal saline at 4C° until processing. Typically, the cord is processed within 12–24 h of birth. The cord is handled in an aseptic fashion and processed in a Type II Biological Safety Cabinet. Surface of the cord is rinsed with sterile phosphate buffered saline to remove as much blood as possible. Length of the cord is estimated. Cord is manipulated in a sterile 10 cm petri dish, cord is cut into 3–5 cm long pieces using a sterile blade. Blood vessels are removed from each piece after incising the cord length wise. Remaining tissue is rinsed. Cord tissue is placed into a sterile 15 ml centrifuge tube and incubated in 3 ml of enzyme solution for 1 hr at 37° C.

After 1 h, the cord pieces are crushed using serrated thumb forceps to release as many cells as possible into the solution. Tissue is moved to a new sterile 10 cm dish filled with phosphate buffered saline, swirled for 5 min, and moved to a new centrifuge tube containing enzyme solution. Tube is incubated for 30 minutes at37° C.

Fig. 1: Human umbilical cord

During this incubation, the centrifuge tube containing solution A is centrifuged at 1000 rpm for 5 min. Supernatant is discarded and 3 ml of medium (see the ''medium'' section) is added to the cell pellet. Cells are re suspended in medium by trituration with a 1000 ml pipette tip to minimize bubble formation and foaming, and the tube is placed in the incubator until the second enzymatic digestion is completed. After the second enzymatic digestion is complete, the cord pieces are squeezed in solution B to remove as many cells from Wharton's jelly as possible. Tube is centrifuged at 1000 rpm for 5 min. The supernatant is discarded and 3 ml of medium is added to the cell pellet. Cells are re suspended in medium by tiurition as in step, cells from the two enzymatic digestion steps are combined. Optional step: Lyse red blood cells.. live cells are counted using a hemocytometer and plated in a 6-well tissue culture plate at a concentration of 30,000 cells per cm^2. Incubate plate at 37°C, 5% CO^2 for 24–72 h. After 24–72 h, the floating cells are transferred to a new plate to allow additional cells to adhere. The cells in the original plate are fed with fresh mediumThe cells are fed by the removal/replacement of half the medium every 2–3 days till the cells reach approximately 80% confluence.

Passaging the cells

Cells are passaged when they are 80–90% confluent. . Medium is aspirated off and the cells are rinsed with sterile phosphate buffered saline (Ca^2free). A minimum amount of warmed, CO2-equilibrated trypsin–EDTA (0.05%) is added to the plate and/or flask to cover the culture surface—0.5 ml to each well of a 6-well plate, 1 ml to a T-25 flask, and 2 ml to a T-75 flask. Plate and/or flask is allowed to sit at room temperature for 1–2 min. Then the detachment of the cells is observed under a microscope and detachment facilitated by repeatedly tapping the plate and/or flask gently on a hard surface. Cells are not allowed to be in contact with trypsin–EDTA for more than 5 min. Trypsinization reaction is neutralized by adding 2–3 times volumes of medium. Solution containing the cells is transferred to a 15 ml sterile centrifuge tube and centrifuged at 1000 rpm for 5 min at room temperature. ix. The supernatant is discarded and the cells are resuspended gently in fresh medium. The cells are counted and transferred to a new plate or flask at a concentration of 10,000 cells per cm2 in fresh medium. Plates and/or flasks are incubated at 37°C, saturating humidity and 5% CO^2. Plates and/or flasks are checked for confluence every day and the cells are fed every other day by removing half the medium and replacing it with
fresh medium. Amount of medium in a plate or flask is as follows: One well of a 6-well plate, 1.5 ml, One T-25 flask, 4 ml and One T-75 flask, 10 ml.

Feeding the cells

Cells are fed every other day or every 3 days. ii. Half the medium in the plate or flask is aspirated off and is replaced with fresh medium.

Counting the cells

Cells are counted by trypan blue exclusion assay using a hemocytometer. This standard method will not be elaborated here (protocol provided upon request).

Cryopreservation

Cells collected for freezing should be in the growth phase. Cells are lifted as described for passaging, except that 4°C freezing medium is added to the cells rather than re suspending them in medium. Cells in the freezing medium are transferred into a cryo vial at 4°C. Cryo vial is transferred to a controlled rate cooler, like Mr. Frosty, maintained at 4°C and placed in the coldest part of the -80°C freezer. Cryo vial is transferred to a liquid nitrogen tank in a day or two. To thaw, the vials are removed from the LN^2 tank and plunged into at 37°C water bath with gentle swirling, before the last ice crystal has melted, the vials are wiped down twice with 70% ethanol and moved to the Bio Safety Cabinet (BSC). Contents of the vial are added slowly with gentle swirling to 5 ml of fresh medium and the vial rinsed twice with medium, the cells are pelleted and re suspended in 1 ml of medium and a live/dead
count made. Cells are plated at 20,000 cells/cm2 for the first passage following thaw.

Materials

Enzyme solutions
Solution A: Collagenase and Hyaluronidase,
a) Collagenase Type I, 300 units/ml
b) Hyaluronidase from ovine testes, 1 mg/ml
c) Phosphate buffered saline with 3 mM $CaCl^2$cell suspension

Solution B: Trypsin-EDTA (0.1%)

Growth Medium

Medium is filtered through sterilized using a 0.22 mm filter and stored refrigerated. Medium is protected from light and prepared fresh weekly.
For small animals, such as rat and mice, it is impractical to remove vessels. In this case, the vessels are not dissected from cord; rather the cord is torn to pieces using sharpened forceps. For small rodents, swine, bovine, and human, small explants created by dicing the cord material with sterile blades may be used as starting material without enzymatic digestion. Start-up is slower than with enzymatically digested material. Standard protocols for lysing red blood cells can be applied and may have a positive effect. This has not been quantified or investigated fully. UCMSCs like high-cell density and start faster when grown at high density; 15,000 cells per square centimeter is recommended. At passage, early passages are at splits of 1:2. Passages 4 and on may be at splits of 1:3 or 1:4. The smallest, round phase bright cells are important for maintaining the culture and are most sensitive to effects of enzyme and other mechanical damage. The cells are sensitive to enzymatic treatment and to rough manipulation. Avoid exposure for more than 5 min to enzymes. If the cells do not lift rapidly, the UCMSCs may have differentiated or senesced. Preliminary work indicates that UCMSCs may prefer 2–5% oxygen tension for culture. This has not been verified in our lab but it agrees with our observation of the cells being sensitive to stressful treatment.As mentioned in results, four different freezing medium have been tested. While good viability is obtained overall, significant variability has been noted at start-up after thawing. This seems to be due to factors involving the freezing (and not the thawing) procedure. The best way to ensure consistency and optimal viability is to prepare small batches of cells for freezing and avoid trying to process many plates at a time. When plating cells after a thaw, if cells do not attach in 24 h and start growing, discard and try a second via1 2. The cells at early passage have typical fibroblast, that is, MSC-like morphology, whereas cells at late passages are elongated and start losing the characteristic morphology of MSCs.

UCMSCs and Culture Characteristics

1. Experiments were carried out to optimize the freezing protocol for human UCMSCs. Cells from nine umbilical cords were frozen at passage 4 and passage 8 in three different freezing media; growth medium served as a negative control. Results from viability at thaw are shown in. From this data, it appears that 90% FBS and 10% DMSO yields about 80% viability; this is more than for the other media tested.

DISCUSSION

Cells from Wharton's Jelly can be isolated using another method called the ''Explant Method.'' For this method, the tissue is chopped into small pieces, about 1 mm^2, and plated with medium. Explants attach to the substrate and the cells outgrow from the tissue. These cells are harvested and passaged. Shortcoming with this method is the inability to determine the number of cells that have been isolated from the cord at passage, because the cells continue to outgrow from the explants even after the cells have been harvested. With the enzymatic digestion method, the number of cells isolated from the umbilical cord is fixed and known ,Determination of population doubling of the cells is possible when the number of cells to start with is known. Several hurdles remain before UCMSC culture is optimal to grow cells for use in human clinical trials. Current culture practices use FBS as a constituent of the medium for UCMSCs, which introduces some limitations. Cells might become infected because of transmission of pathogens (prions and virus) from the serum and/or adherence of animal protein molecules to the cell surface, causing immune reactions. There is a possibility for the cells to get contaminated during culture. If the patients' safety is compromised, the cells cannot be used to treat diseases in spite of their potential. Also, batch to batch variability may be seen even if the serum is procured from the same vendor. This variability may affect the growth of the cells and the time for which they remain in the undifferentiated state. Therefore, to use the cells in clinical trials, they have to be grown in an appropriate serum-free medium. Isolation of cells is done manually and is time consuming (3–4 h per cord). This is another hurdle in generating clinical grade cells. To be able to use the cells in clinics, the isolation procedure ideally has to involve minimal tissue handling and be less time consuming.

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