PCCS Growth Media, Cell Tagging, Cell Separation Final Assignment

Panama College of Cell Science

In partial fulfillment of the requirements of course 646

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Choice Number 2: This choice, if you select it, requires you to answer a series of problems (questions) on different topics germane to expansion protocols. Please study the **RULES section** above for guidance in answering these questions. For this Choice Number 2, you must answer **all** of the following questions:

You may select as your starting point 50 ml of patient's whole blood or 25 ml of aspirated fatty tissue. Once you state which cells are your starting point, ALL of the following questions must be answered as to that type of cells only.

A. Select hematopoietic stem cells from blood or mesenchymal stem cells from fatty tissue:
____X____Hematopoietic _____Mesenchymal

B. Cell Separation: From the starting point in part A above, please state a cell separation technique you could use to separate out and collect only the stem cells. Your answer must include the equipment you would use, and your answer must include the number of stem cells collected.

C. Cell Tagging: What are some labeling techniques (for example, fluorescent antibody) you would use to verify you are counting stem cells or to verify you have collected stem cells after separation? Where will you purchase such material? Be specific.

D. Growth Media: What growth media will you propose to use that will least contaminate the process with animal components, and will be best for use of the expansion product in a clinical setting?

E. Batch Processing: What procedure will you recommend to yield a significant increase in the number of stem cells, and what number of stem cells will you expect to have after expansion?

Having 50mL of blood available and, in order to separate hematopoietic stem cells from it, I would use the CD34 MicroBead Kit UltraPure, human from Miltenyl Biotec. This kit is suitable for positive selection of CD34+ hematopoietic stem cells and suited for all routine CD34 cell isolations; this kit is ideal for those samples which are debris-rich and low frequency [1]. The protocol for the separation of hematopoietic stem cells using the CD34 MicroBead Kit UltraPure requires the following reagents and instruments:

- A solution containing: phosphate-buffered saline (PBS), pH 7.2, 0.5% bovine serum albumin (BSA) and 2mM EDTA. This is achieve by diluting MACS BSA Stock solution 1:20 with autoMACS Rinsing Solution. Buffer should be cold (2 - 8°C). Before usage buffer should be degas to prevent air bubbles entering the column [1].
- MACS Columns and MACS Separators: CD34+ cells can be enriched by using one of the following MS, LS or XS Columns. For the purpose of the experiment the column to be used is MS since the maximum number of cells labeled will be 10^7 and for the available volume of blood (50mL), less than 2 x 10^6 will be collected [2].

Sample preparation is as follows:

- It is necessary to isolate peripheral blood mononuclear cells (PBMCs) using a density gradient centrifugation (i.e. Ficoll-Paque) [1].
- Platelets are removed by resuspending the pellet in buffer and centrifuge at 200g for a period of 10 – 15 minutes at 20°C, supernatant is aspirate, wash step is repeated [1].

After this step, cells are magnetic labelled as follows:

- Cells should be keep cold, solutions pre-cooled and work fast ensuring safety and precautions [1].
- Before magnetic labelling a single-cell suspension is important. Cells are pass through a 30µm nylon mesh in order to remove cell clumps, this way cell clumps are removed ensuring a proper function of the column [1].
- Incubation temperature is 2 - 8°C [1].
- Cell number is determine [1].
- Cell suspension is centrifuge at 300xg for 10 minutes; supernatant is completely aspirate [1].
- Cell pellet is resuspended in 300µL of buffer for up to 10^8 cells (total) [1].
- Add 100µL of FcR Blocking Reagent for up to 10^8 cells (total) [1].
- Add 100µL of CD34 MicroBeads UltraPure for up to 10^8 cells (total) [1].
- Mix and incubate for 30 minutes in the refrigerator at a temperature of 2 - 8°C [1].
- As an optional step, fluorochrome-conjugated CD34 antibody can be added, incubate for 5 minutes in the dark at a temperature of 2 - 8°C [1].
- Cells are wash adding 5 – 10 mL of buffer for up to 10^8 cells and centrifuge at 300xg for 10 minutes; supernatant is completely aspirate[1].
- Cells are resuspended (up to 10^8) in 500µL of buffer [1].
- Cells are ready for magnetic separation [1].

Cells are separated using MACS columns. For the purpose of this experimentation the column to be used is the model MS which have a capacity of $2x10^8$ total cells and a maximum of labeled cells of 10^7 [1]. The procedure is as follows:

- Column is placed in the magnetic field of a suitable MACS Separator [1].
- Column is prepare by rinsing it with 500µL of buffer [1].
- Cell suspension is applied to the column. Flow-through contains unlabeled cells and it is collected [1].
- Column is washed using 3 x 500µL. Unlabeled cells are collected and combined with the flow through from the previous step [1].
- Column is remove from the separator and place on a suitable collection tube [1].
- Pipette 1 mL of buffer onto the column. Flush out the magnetically labeled cells by firmly pushing the plunger into the columns [1].
- To increase the purity of the CD34+ cells the eluted fraction can be enriched on a second MS column (this step is optional) [1].

The purity of these cells can be evaluated using flow cytometry or fluorescence microscopy. CD34+ cells can be analyze by direct immunofluorescence staining using an antibody recognizing an epitope different from that recognized by the CD34 monoclonal antibody QBEND/10 [1]. Cells are stained for 5 minutes at 2 - 8°C; after staining, cells should be washed and resuspend in buffer [1]. Miltenyl Biotec has a flow cytometer called MACSQuant Analyzer 10. It is a powerful benchtop flow cytometer which have a high sensitive, multi-parameter cell analysis. It is fully automated; equipped with three lasers (405, 488 638 nm), two scatter (FSC, SSC) and eight fluorescent channels allowing to have a rapid, full automated multi-color flow cytometry [3].

Cells can be also counted using a hematocytometer. It can be used as follows; In order to prepare 1 mL of the cell sample the analyst or scientist has to add 760 µL of the medium, 140 µL of Trypan blue and 100 µL of cell sample. Trypan blue is used to detect dead cells in the culture. When cells are dead the membrane become permeable, Trypan blue enters the cell and it becomes blue, this way dead cells are not counted. After the sample preparation, the hemacytometer and the slide is cleaned using 75% ethanol; analyst has to ensure the hemacytometer and the slide are completely dry before injecting the sample. Slide is placed in the hemacytometer. 10 µL of the sample is placed in both wells of the hemacytometer. Under the microscope it can be noticed that the hemacytometer will be divided into quadrants or squares (could be 4 or 5), the volume in each quadrant is 0.1 µL. Cells are counted per quadrant.

After the cells are counted it is time to perform the necessary calculations to determine the total number of cells per mL of sample. Important to keep in mind that 0.1 µL equals 0.0001 mL, thus 1 µL is 1/1000 mL. First, the average number of cells per square (number of cells per 0.1 µL) has to be determined. This number is multiply by the original dilution factor to obtain the concentration of cells per µL. Then this number is multiply by 1000 to obtain the concentration of cells per mL. If is required, the concentration of cells per mL is multiply by the total volume of the cell suspension to obtain the total number of cells in it.

The hemacytometer is a relatively easy instrument to use, but it is important to ensure that the use and the calculations are understood and dominated in order to have an accurate count of cells. If both skills are not mastered, the percentage of error when performing this exercise will be high.

The expected range of stem cells that it can be separate from 50 cc of the patient's blood is led than 2 x 10^6 [2].

For expansion purposes I would definitely recommend autologous serum and plasma from the same patient as the growth medium. This will ensure that there will be no animal components present on the culture. According to Bieback et al "for clinical scale manufacturing, human factors from serum or platelets have been suggested" [4].

Endothelial cells are the basic building blocks of the vascular system [5]. These cells form the inner liming of blood vessels and also provide an anticoagulant barrier between the vessel wall and blood [5]. They played a role as a selective permeability barrier; they are multifunctional cells with critical basal and inducible metabolic and synthetic functions [5]. Endothelial cells regulate homeostasis, vasomotor tone, and immune and inflammatory responses; coagulation, growth regulation, production of extracellular matrix components and blood flow modulator [5].

I would use the "buckets of stem cells" approach, co-culturing the hematopoietic stem cells along with endothelial cells. Of course always maintaining the cells in autologous serum and plasma. According to Butler et al. by co-culturing endothelial cells along with stem cells researchers were able to manufacture an unlimited supply of blood-related stem cells enabling patients to have bone marrow available whenever they needed. Endothelial cells also helped grow stem cells such as brain, heart, skin and lungs [6] without the addition of growth factors and serum. The growth factors stem cells would need are provided by the endothelial cells. Researchers also discovered that endothelial cells also helped to instruct the stem cells to generate mature differentiated progeny with the potential to form immune cells, platelets, red cells and white cells by expressed stem cell active cytokines.

Now, if I started with 2 x 10^6 cells and, according to the report, these endothelial cells can boost expansion capability of human adult stem cells by 400 fold, I would end up with approximately 8 x 10^8 cells (80,000,0000). In order to achieve the therapeutical number of stem cells (1 x 10^9), a serial collection of blood can be performed.

Having the technology and the right conditions this could be a very successful experiment. I believe the answer is there; if scientist focus on the use of autologous serum and plasma, animal contamination and other issue can be ruled out and a real therapeutic alternative can be offered to every patient.

References:

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