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CHARACTERIZATIONS OF CD34+ STEM CELLS FROM HEMATOPOIETIC STEM CELLS (HSCS)

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ABSTRACT

Stem cells are unspecialized cells in the human body that are capable of becoming specialized cells, each with new specialized cell functions. Hematopoietic stem cells (HSCs) originated from mesodermal hemangioblast cells which have an ability to self-renew and multipotent nature. These HSCs usually express CD34+ and CD133+ glycoproteins on their surface and antibodies raised against these surface glycoproteins are the best exploited matter in the purification steps. $CD34^+$ is a cluster of differentiation molecule presents on certain cells within the human body. The present research mainly focused on characterization of CD34+ stem cells from HSCs, which is in associated with diagnose of varius tumors such as alveolar soft part sarcoma, AML-M7 and preB-ALL. CD34 cells were maintained in the laboratory under appropriate conditions. The cells were cultured in DMEM with 95% humidity and 5% CO2. They showed round shaped actively dividing state under daily observations. These cells were stained with Leishman-Giemsa (LG) stain showed nucleated structures with no contamination with other morphologically dissimilar structures. The expression of CD34 surface glycoprotein in the growing cells was confirmed by immunocytochemistry (ICC). For detection of CD34 marker on the cultured cells, the Anti CD34 antibody was used and probed against anti-mouse HRPO sterpavidin conjugate as secondary antibody. The secondary antibody when added with developer, DAB-H_2O_2 given brown Colour precipitate colony forming unit assay showed efficiency that $CD34^+$ stem cells can be determined based on number of colonies which emerge. This is based on the ability of cells to grow unattached and to remain suspended in agar. The results showed the presence of $CD34^+$marker where the AntiCD34 antibody binds specifically to the $CD34^+$ marker present on the cell surface of hematopoietic stem cells.

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INTRODUCTION

Stem cells are defined functionally as cells that have the capacity to self-renew as well as the ability to generate differentiated cells (Weissman *et al*., 2001). The best example of a stem cell is the bone marrow stem cell that is unspecialized and able to specialize into blood cells, such as white blood cells and red blood cells, and these new cell types have special functions, such as being able to produce antibodies, act as scavengers to combat infection and transport gases. Thus one cell type stems from the other and hence the term "stem cell (Hans, 2007). Based on the potentiality it can be differentiated. Stem cells are divided into embryonic stem cells, fetal stem cells, Amniotic stem cells, Induced pluripotent stem cells, hematopoietic stem cells (ArirT Bongso, 2005).

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Hematopoietic stem cells (HSCs) originated from mesodermal hemangioblast cells which have an ability to self-renew and multipotent nature (Dzierzak, 1999). HSCs may found in the bone marrow, peripheral blood, and umbilical cord blood with a range of 0.03-0.09% to the total leukocyte count (Dzierzak *et al*., 1998 and Huyhn *et al*., 1995). In adult body these HSCs migrate from bone marrow to peripheral blood through action of various cytokines, such as granulocyte-colony stimulating factor (G-CSF), Granulocyte macrophage-colony stimulating factor (GM-CSF) etc (Perkins, 1998). This induction through G-CSF or GM-CSF triggers expression of specific matrixmetallo proteases from neutrophils which can break the interaction between SDF1 and CXCR4 leading to release of these cells into peripheral blood stream and also initiating proliferation of HSCs (Hole *et al*., 1999). These HSCs usually express CD34+ and CD133+ glycoproteins on their surface and antibodies raised against these surface glycoproteins are the best exploited in the purification steps. Although apheresis techniques has been standardized long ago still the preparation

shows the distinct presence of niesenchymal stem cells, stromal cells along with HSCs, with advent of FACS in the purification of cells it will now possible to obtain pure HSCs. In this FACS technique fluorescent labelled CD34 antibodies are used to sort out CD34+ stem cells (He *et al*., 1992). Such pure homogenous stem cells can be used to grow in vitro culture medium like DMEM and thus cultured cells can be induced with various compounds to differentiate into different cell types enabling us to understand the differentiation and organization of cells into tissues. Therefore, such homogenous preparations can be used in the autologous transplantation for the repair of damaged cardiac tissue due to severe ischemia, neurodegenerative diseases, spinal cord injuries etc.

To identify hematopoietic stem cells, $CD34^+$ used as marker. $CD34^+$ is a cluster of differentiation molecule present on certain cells within the human body (Simmons *et al*., 1992). It is present on the progenitor cells of hematopoietic stem cells it get disappeared in differentiated cells. It is a cell surface glycoprotein and functions as a cell-cell adhesion factor. It may also mediate the attachment of stem cells to bone marrow extracellular matrix or directly to stromal cells. The $CD34^+$ protein is a member of a family of single-pass transmembrane sialomucin proteins. The $CD34^+$ gene is located on chromosome Iq32.cd34 (Satterthwaite *et al*., 1992), mainly plays a key role in promoting lymphocyte adhesion and blocking adhesion. $CD34^+$ cells differentiate to various tissues according to" the growth factors they utilized. In recent findings states that $CD34^+$ cells differentiated to non hematopoietic tissue also (eg: hepatocytes in liver) (He *et al*., 1992). $CD34^+$ was used to cure Graft-Versus-Tumor Treatment of Cancer, Cancer Chemotherapy, Inherited Blood Disorders, Leukemia and Lymphoma (Doyonnas *et al*., 2001; Brown *et al*., 1991 and Sassetti *et al*., 2000).

MATERIALS AND METHODS

Isolation of $CD34^+$ cells from HSCs

Culturing of $CP34^+$ Cells: HEPES was weighed in a sterile foil and dissolved in autoclaved distilled water and pH. is adjusted to 7.4 with NaOH. To the above solution NaHC03 was added followed by different antibiotics (Table 1) which serve as bacteriocidies and fiingicicides were added to the DMEM medium. Human adults stem cells isolated from a volunteer in 2006 were stored in the -86°C. These cells revived and allowed to grow in DMEM medium containing 10% FCS (Fetal calf serum) in a carbon dioxide incubator set at 5% CO2, 95% humidity and 37°C (Sarma and Subramanyam, 2008). These freshly grown cultures were used for the characterization of CD34 protein.

Table 1. Table showing concentrations of Antibiotics

Antibiotics	Stock	Working stock
Amphotericin-B	50mg/ml	2.5µg/ml
Ampicillin	100mg/ml	5 µg/ml
Gentamycin	25 µg/ml	100 µg/ml
Kanamycin	25mg/ml	100 µg/ml
Streptomycin	25mg/ml	10 µg/ml
Tetracyclin	25 mg/ml	10 µg/ml

Cell viability assay

Equal volumes of CD34+ cell suspension and 0.4% Trypan blue dye (w/v) was mixed and counted in improved Neubauer haemocytometer within 2-3 minutes; avoid longer incubation periods to reduce viability count. The sample with >85% viability was considered as acceptable criterion for further characterization.

$$\% \ of \ \text{cell Viability} = \frac{\text{Total number of viable cells}}{\text{Total number of cells}} X \ 100$$

Identification of $CD34^+$ cells by Immunocyto chemistry (ICC)

ICC was performed to observe the presence of CD34 antigen in growing human adult stem cells, present in a given sample or not by using the primary and secondary antibodies (anti-cd34antibody and anti-mouse HRPO) (Codias, 1990; Howell *et al*., 1991). A clean and dry glass slide was taken and to this added 10µl of growing culture spreaded into smear. Allowed to dry, after fixed the smear with 4%paraformaldehyde and leaved it at 4°C for lhr. Washed the slide with several times with PBS until no pungent smell was observed. Soaked the slide in 3%H2O2, and kept it in room temperature for 30min. washed the slide with PBS 3 to 4 times and added blocking buffer and leave at 37°C for 30min. washed the slide with PBST until no air bubbles were seen. Then added primary antibody and incubated at 37°C for l hr. (Anti-CD34 Ab). Then washed the slide with PBST for one time and PBS with two times. Now added 1:400 ratio anti-mouse HRPO sterpavidin conjugate as secondary antibody and incubated at 37C for lhr. Washed with PBST for 3times and last 2times with 10mM Tris PH-8. Finally now added developer and leave it in dark at room temperature for 30 to 60min. After incubation time brown colour appeared in slide, soaked the slide in distilled water for 2min and air dried, observed the slide under microscope.

Colony Forming Unit Assay (CFU Assay) for Self-Renewal

Prepare stock of 1.8% noble agar in sterile deionized water

Add 1.8 g noble agar to 100ml deionized water. Autoclave at 121°C for 15 min. Keep this solution in a 55°C water bath until ready to use.

Prepare 2X DMEM

IX DMEM powder (originally intended to make 1L solution) was added into 500ml deionized water. Sterile filter this solution and place at 37°C (to prevent premature solidification of noble agar when noble agar is added).

Prepare Stem cells

Wash Stem cell cultures twice in IX PBS. Detach cells using trypsin-EDTA, harvest via centrifugation, and count cells. Dilute appropriately to add logl 0-fold dilutions of cells to 1.33ml sterile PBS in microcentrifuge tubes. For example, add 1 cell to a tube with 1.33ml PBS, add 10 cells to a tube with

1.33ml PBS, add 100 cells to a tube with 1.33ml PBS, etc. 3d. Set this cell solution aside until ready to seed (see step 5).

Plate the bottom agar into 60mm Petri dishes

In a sterile 10ml tube, mix 2ml of 2X DMEM with 1.33ml of 1.8% noble agar and 0.67ml sterile deionized water. Immediately pour this 4ml solution onto 60mm Petri plates. Perform in triplicates. 4c. Allow plates to solidify at 37°C for 10 min.

Plate the top agar

In a sterile 10ml tube, Mix 2ml of 2X DMEM with 0.67ml of 1.8% noble agar and 1.33ml cell solution from Step 3c). Immediately pour this 4ml solution onto the bottom layer of 60mm Petri plates from Step. Perform in triplicates. Place plates at 37°C overnight to allow for colony formation. Using a Inverted microscope, count the number of colonies on each 60mm plate. Determine the efficiency based on the number of cells plated: Efficiency = (# of colonies) / (# of cells plated) x 100%

RESULTS

CD34 cells were maintained in the laboratory under appropriate conditions. The cells were cultured in DMEM with 95% humidity and 5% CO2. The cells showed round shaped actively dividing state under daily observations. These cells were stained with Leishman-Giemsa (LG) stain showed nucleated structures with no contamination with other morphologically dissimilar structures. The expression of CD34 surface glycoprotein in the growing cells was confirmed by immunocytochemistry (ICC). For detection of CD34 marker on the cultured cells the Anti CD34 antibody jwas used and probed against anti-mouse HRPO sterpavidin conjugate as secondary antibody. The secondary antibody when added with developer, DAB-H_2O_2 given brown Colored precipitate. Hence, the $CD34^+$ marker was characterized in the present study. The Colony Forming Unit Assay showed efficiency of $CD34^+$ stem cells can be determined based on number of colonies that emerge. This is based on the ability of cells to grow unattached and to remain suspended in agar. Secondly, the self-renewal properties of Stem cells can be assessed based on the types of progeny produced in noble agar.

DISCUSSION

Stem cells have capability to become self renew or differentiation. These stem cells differentiate based on their potentiality. In these hematopoietic stem cell is the common ancestor of all blood cells. Hematopoietic stem cell involved in tissue and bone remodeling and removal of dead cells (Reya, 2003). Many blood cells are short lived and need to be replenished continuously. The average human requires approximately one hundred billion new hematopoietic cells each day. HSCs progenitors are small quiescent cells that express the surface glyco protein $CD34^+$. $CD34^+$ hSCs undergoes differentiation and form the blood cells. $CD34^+$ is also used as a marker for identification and isolation of HSCs and tissue specific stem cells. $CD34^+$ protein has the serine, threonine and proline rich extra cellular domain that is extensively o-glycosylated and sialylated (Li *et al*., 2001; Li *et al*., 2001). $CD34^+$is a cell surface Trans membrane protein promotes the proliferation and blocking differentiation. The discovery of the sialomucin CD34 as a hematopoietic cell surface antigen has transformed and accelerated studies on human hematopoietic development. Cell surface expression of the CD34 antigen has rapidly become the distinguishing feature used as the basis for enumeration, isolation, and manipulation of human stem cells, because CD34 is downregulated as cells differentiate into more-abundant mature cells eg: endothelial cells, myogenic progenitor cells, hepatocytes etc. Hematopoietic stem cell transplantation (HSCT) has become the standard of care for the treatment of several spinal disorders, infracted cardiac tissue and other blood disorders etc. Recruitment of hematopoietic stem/progenitor cells into the peripheral blood involves with induction of certain cytokines. Cytokine therapy mimics the enhancement and physiological release of stem cells into the peripheral blood from the bone marrow reservoir. This mobilization process is initiated by stress-induced activation of neutrophils causes the release of proteolytic enzymes which sheds the migration and proliferation of HSCs into the peripheral blood (Sharp *et al*., 2000; Greenwood *et al*., 2003 and Wright *et al*., 2001).

Summary and Conclusion

$CD34^+$ cells are said to be the marker for Hematopoietic stem cells which are highly beneficial to develop multi lineages, need to characterize the CD34 marker is predominant. In the present study the Characterization was carried out by the methods involving Immunocyto chemistry, CFU Assay. The results showed the presence of $CD34^+$marker where the AntiCD34 antibody binds specifically to the $CD34^+$ marker present on the cell surface of Hematopoietic stem cells.

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