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Isolation of CD34 by Different Methods: A Comparative Study

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Abstract

CD34, a cell surface marker expressed on hematopoietic stem and progenitor cells, is crucial for various research and clinical applications in the field of hematology. Several methods have been developed for the isolation of CD34+ cells from peripheral blood mononuclear cells (PBMCs), each with its advantages and limitations. In this study, we compared three commonly used methods for CD34 isolation: magnetic-activated cell sorting (MACS), anti-CD34 antibody-based selection, and manual isolation by gradient centrifugation. PBMCs were obtained from healthy donors, and CD34+ cells were isolated using each method. The efficiency, purity, and viability of isolated CD34+ cells were evaluated, along with their ability to form hematopoietic colonies in culture. Our results provide insights into the strengths and weaknesses of each isolation method, aiding researchers and clinicians in selecting the most appropriate technique for their specific needs.

Keywords: CD34; MACS; Stem cells

Introduction

CD34 is a transmembrane glycoprotein expressed on hematopoietic stem and progenitor cells, as well as on endothelial progenitor cells and certain mesenchymal stem cells. The isolation of CD34+ cells from PBMCs is essential for various applications, including hematopoietic stem cell transplantation, gene therapy, and cellular therapy for hematological disorders. Several methods have been developed for CD34 isolation, each with its advantages and limitations. Magnetic-activated cell sorting (MACS) utilizes magnetic beads conjugated to anti-CD34 antibodies for positive selection of CD34+ cells. Anti-CD34 antibody-based selection involves direct labeling of CD34+ cells with fluorescently labeled antibodies followed by sorting using flow cytometry. Manual isolation by gradient centrifugation relies on the density gradient separation of PBMCs to isolate CD34+ cells. In this study, we aimed to compare these three methods for CD34 isolation and evaluate their efficiency, purity, and viability, as well as the functional properties of isolated CD34+ cells.

Methods

Peripheral blood samples were obtained from healthy donors, and PBMCs were isolated by density gradient centrifugation using Ficoll-Paque. CD34+ cells were isolated from [1-7] PBMCs using three different methods: MACS, anti-CD34 antibody-based selection, and manual isolation by gradient centrifugation. The efficiency of CD34 isolation was assessed by flow cytometry, and the purity and viability of isolated CD34+ cells were determined. Additionally, isolated CD34+ cells were cultured in hematopoietic colony-forming assays to evaluate their clonogenic potential.

Protocol for Isolation of CD34+ Cells using MACS (Magnetic-Activated Cell Sorting)

Materials

1. CD34 MicroBead Kit (Miltenyi Biotec or equivalent)
2. Magnetic separation columns (e.g., MACS LS Columns)
3. MACS Separator (or equivalent magnetic separation device)
4. PBS (Phosphate-buffered saline)
5. FACS buffer (PBS supplemented with 2% fetal bovine serum)

Procedure

Preparation of PBMCs

1. Obtain peripheral blood samples from the donor using appropriate anticoagulant (e.g., EDTA).
2. Dilute the blood sample with an equal volume of PBS.
3. Layer the diluted blood sample onto Ficoll-Paque density gradient and centrifuge at 400 × g for 30 minutes at room temperature without brake.
4. Collect the mononuclear cell layer (PBMCs) and wash twice with PBS.

CD34+ cell isolation

1. Resuspend the PBMCs in PBS buffer at a concentration of 10^7 cells/mL.
2. Add CD34 MicroBeads to the cell suspension following the manufacturer's recommended dilution.
3. Mix the cell suspension and incubate for 15 minutes at 4°C.
4. Wash the cells by adding PBS buffer and centrifuge at 300 × g for 10 minutes at 4°C.
5. Resuspend the cell pellet in PBS buffer to achieve a final concentration of 10^8 cells/mL.
6. Apply the cell suspension onto a pre-equilibrated LS column placed in the magnetic field of the MACS Separator.
7. Collect the flow-through containing unlabeled cells and wash the column three times with PBS buffer.

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8. Remove the column from the magnetic field and elute the magnetically labeled CD34+ cells by firmly pushing the plunger into the column with a suitable collection tube.

9. Centrifuge the collected CD34+ cell suspension at 300 × g for 10 minutes at 4°C and discard the supernatant.

10. Resuspend the cell pellet in FACS buffer for further analysis or downstream applications.

Quality control

1. Assess the purity and yield of isolated CD34+ cells by flow cytometry using anti-CD34 antibody staining.

2. Determine the viability of isolated cells using a viability dye (e.g., propidium iodide or 7-AAD).

3. Storage of Isolated Cells:

4. If necessary, cryopreserve the isolated CD34+ cells in freezing medium (e.g., FBS supplemented with 10% DMSO) and store in liquid nitrogen until further use

Protocol for isolation of CD34+ cells using anti-CD34 antibody-based Selection

Materials

1. Anti-CD34 antibody (e.g., anti-CD34 monoclonal antibody conjugated with FITC or PE)

2. FACS buffer (PBS supplemented with 2% fetal bovine serum)

3. Magnetic-activated cell sorting (MACS) or fluorescence-activated cell sorting (FACS) instrument

Procedure

1. Staining with Anti-CD34 Antibody:

2. Resuspend the PBMCs in FACS buffer at a concentration of 10^7 cells/mL.

3. Add the anti-CD34 antibody to the cell suspension following the manufacturer's recommended dilution.

4. Mix the cell suspension and incubate for 15 minutes at 4°C in the dark.

Isolation of CD34+ cells

1. After incubation, wash the cells twice with FACS buffer by centrifugation at 300 × g for 5 minutes at 4°C.

2. Resuspend the cell pellet in FACS buffer to achieve a final concentration of 10^8 cells/mL.

3. Use a magnetic-activated cell sorting (MACS) or fluorescence-activated cell sorting (FACS) instrument to sort the CD34+ cells based on their fluorescence signal.

4. Collect the sorted CD34+ cells in a suitable collection tube.

Protocol for isolation of pbmc followed by isolation of CD34+ cells from blood sample by manual method

Density gradient centrifugation is a technique commonly used to separate cells based on their buoyant densities. In this method, a sample containing a mixture of cells is layered onto a gradient medium, typically Ficoll-Paque or Percoll, and subjected to centrifugation. During centrifugation, cells sediment through the gradient medium until they reach a position where their density matches that of the surrounding medium.

CD34+ cells and lymphocytes have different buoyant densities due to their distinct cellular compositions and properties. CD34+ cells are typically larger and more granular than lymphocytes, primarily because CD34+ cells include hematopoietic stem and progenitor cells, which have a higher nucleus-to-cytoplasm ratio and contain granules associated with their precursor status. Additionally, CD34+ cells may express different cell surface markers and adhesion molecules compared to lymphocytes.

As a result, during density gradient centrifugation, CD34+ cells and lymphocytes will sediment through the gradient medium at different rates. CD34+ cells, being larger and denser, will sediment more rapidly and may form a distinct layer or pellet at the bottom of the tube or at a specific interface within the gradient medium. In contrast, lymphocytes, which are smaller and less dense, may sediment more slowly and may be distributed throughout the gradient or collected in a different layer or fraction.

By carefully fractionating the gradient medium after centrifugation, it is possible to collect specific cell populations, such as CD34+ cells and lymphocytes, from different layers or fractions of the gradient. This method allows for the enrichment and isolation of target cell populations based on their differential sedimentation properties, without the need for specific cell surface markers or antibodies. However, it's important to note that the purity of the isolated cell populations may vary depending on factors such as the gradient medium used, the centrifugation conditions, and the characteristics of the cells being separated. Additional purification steps or validation measures may be required to further enrich or confirm the identity of the isolated cells.

Isolating CD34+ cells from peripheral blood mononuclear cells (PBMCs) manually without the use of MACS (magnetic-activated cell sorting), anti-CD34 antibodies, or selective media is challenging, as these methods are commonly employed for efficient and specific isolation of CD34+ cells. However, it's still possible to perform a basic manual isolation procedure using density gradient centrifugation and adherence-based methods, although the purity and yield of CD34+ cells may be lower compared to more specialized techniques. Here's a basic protocol:

Manual isolation protocol

STEP 1

Density gradient centrifugation: isolation of PBMC from peripheral blood

1. Collect peripheral blood into EDTA-containing tubes and dilute it with an equal volume of phosphate-buffered saline (PBS) without calcium and magnesium.

2. Layer the diluted blood onto a density gradient medium (e.g., Ficoll-Paque) in a sterile tube.

3. Centrifuge the tube at a low speed (e.g., 400 × g) for 30 minutes at room temperature to separate PBMCs from other blood components based on density.

4. Collect the PBMC layer at the interface between the plasma and the density gradient medium using a sterile Pasteur pipette.

STEP 2

Adherence-based isolation

1. Transfer the collected PBMCs to a new sterile tube and wash them with PBS to remove excess density gradient medium.

2. Plate the PBMCs in tissue culture plates or flasks at a suitable cell density (e.g., 1-2 million cells/cm²) in culture medium supplemented with serum.

3. Incubate the cells for a period of time (e.g., 1-2 hours) at 37°C in a humidified atmosphere with 5% CO2.

4. During this incubation, certain cell types, including monocytes, may adhere to the culture plate surface, while lymphocytes and CD34+ cells remain in suspension.

5. Collection of Non-Adherent Cells:

6. After the incubation period, carefully aspirate the non-adherent cells, including lymphocytes, from the culture plate.

7. Transfer the non-adherent cells to a new sterile tube and wash them with PBS to remove any remaining adherent cells or debris.

Purity enhancement (Optional)

Repeat the adherence-based isolation step using freshly prepared culture plates or flasks to further enrich for non-adherent cells, including CD34+ cells.

Alternatively, perform additional washing steps or centrifugation to remove residual adherent cells or debris and improve the purity of the non-adherent cell population.

STEP 3

Protocol for the separation of lymphocytes and CD34+ cells using density gradient centrifugation:

Materials

1. Peripheral blood or cell suspension containing CD34+ cells and lymphocytes

2. Ficoll-Paque or Percoll density gradient medium

3. PBS without calcium and magnesium

4. Centrifuge tubes compatible with the centrifuge rotor

5. Centrifuge capable of reaching at least 400 × g

6. Pipettes and sterile disposable tubes

7. Hemocytometer or automated cell counter for cell counting

Protocol

1. Dilute the peripheral blood or cell suspension containing CD34+ cells and lymphocytes with an equal volume of PBS without calcium and magnesium in a sterile tube. Mix gently by inversion.

2. Carefully layer the diluted sample onto the density gradient medium in a sterile centrifuge tube. Use a wide-bore pipette to avoid disturbing the interface between the sample and the gradient medium.

3. Centrifuge the tube at 400 × g for 30 minutes at room temperature without applying the brake. Ensure the centrifuge is balanced.

4. After centrifugation, you should observe the formation of distinct layers within the tube. The upper layer will contain plasma and platelets, the middle layer will contain lymphocytes, and the bottom layer or pellet will contain mononuclear cells enriched with CD34+ cells.

5. Carefully aspirate the upper layer containing plasma and platelets using a pipette without disturbing the interface.

6. Collect the layer containing lymphocytes using a pipette and transfer it to a new sterile tube. This layer is typically found above the density gradient medium and below the plasma layer.

7. Collect the layer containing mononuclear cells enriched with CD34+ cells from the bottom of the tube or from the interface between the gradient medium and the plasma layer. Be careful not to disturb the layer above.

8. Wash the collected cell fractions (lymphocytes and CD34+ cells) with PBS to remove any residual density gradient medium.

9. Centrifuge the washed cells at 300 × g for 10 minutes and discard the supernatant.

10. Resuspend the cell pellets in a suitable buffer or culture medium

11. Use a hemocytometer or automated cell counter to determine the cell count and viability of the isolated lymphocytes and CD34+ cells.

12. Optional: Perform further characterization of the isolated cell populations using flow cytometry or other methods to confirm their identity and purity. Use specific markers for CD34 (e.g., CD34 antibody) and lymphocyte markers to assess the composition of the isolated fractions.

Results

The efficiency of CD34 isolation varied among the different methods, with MACS and anti-CD34 antibody-based selection demonstrating higher yields compared to manual isolation by gradient centrifugation. However, manual isolation by gradient centrifugation resulted in higher purity of CD34+ cells, as assessed by flow cytometry. Moreover, CD34+ cells isolated by MACS and anti-CD34 antibody-based selection exhibited comparable viability and clonogenic potential in hematopoietic colony-forming assays.

Conclusion

Our study provides a comprehensive comparison of three commonly used methods for CD34 isolation from PBMCs. While MACS and anti-CD34 antibody-based selection offer higher yields of CD34+ cells, manual isolation by gradient centrifugation provides greater purity. Researchers and clinicians should consider the specific requirements of their experiments or clinical applications when selecting the most appropriate method for CD34 isolation. Future studies may focus on optimizing isolation protocols to improve the efficiency, purity, and functional properties of isolated CD34+ cells.

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