Chemically defined cytokine-free expansion of human haematopoietic stem cells

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Method Article

Keywords:

Posted Date: March 27th, 2023

DOI: https://doi.org/10.21203/rs.3.pex-2163/v1

EDITORIAL NOTE:

The original text of this protocol contained an error. In the Reagents section it read: "Add **10 mg** of PVA or Soluplus® to a sterile flask and increase the solution to 100 ml with sterile water."
The correct instruction is: "Add **10 g** of PVA or Soluplus® to a sterile flask and increase the solution to 100 ml with sterile water."
The text was corrected on 25 July, 2023.

Abstract

The ex vivo expansion of human hematopoietic stem/progenitor cells (HSPCs) has important applications in both basic research and clinical transplantation therapy. Here, we introduce the protocol of a novel culture system that supports the long-term ex vivo expansion of human HSPCs, achieved through the complete replacement of cytokines and albumin by chemical agonists and a caprolactam-based polymer. A phosphoinositide 3-kinase activator in combination with a thrombopoietin receptor agonist and the pyrimidoindole derivative UM171 were sufficient to stimulate functional expansion of umbilical cord blood-derived HSCs. We envision that this chemically defined expansion culture system will help to advance clinical HSC therapies.

Introduction

Hematopoietic stem cells (HSCs) are a rare cell population that reside in the adult bone marrow (BM) and support life-long hematopoiesis, through their ability to both self-renew and differentiate into all mature blood cell types (1-4). These activities also support hematopoietic system reconstitution following HSC transplantation (HSCT), which is a curative treatment for a variety of hematological diseases (5). HSCs can also be collected from umbilical cord blood (CB), which represents a highly accessible source for transplantation but are often in insufficient numbers to support successful engraftment and durable hematopoietic system reconstitution (6). Ex vivo expansion of human HSCs, particularly CB-derived HSCs, is therefore a major goal in hematology and one that remains a substantial barrier to the wider and safer therapeutic use of HSC therapies (7). While various reagents have been tested in our attempts to grow human HSCs, cytokines have long thought to be essential for supporting HSCs ex vivo (8). Various recombinant cytokines are commonly added to human HSC cultures, usually in combination with serum albumin. These cultures generally support short-term maintenance of HSCs ex vivo but do not expand functional HSCs. More recently, two-week ex vivo expansion of human HSCs has been achieved by the addition of small molecules, StemRegenin 1 (SR-1) (9) and UM171 (10), to cytokine-supplemented media, and clinical trials using these approaches to expand CB HSCs prior to transplantation reporting encouraging results (11, 12). Other recent approaches have included use of 3-dimensional zwitterionic hydrogels (13), addition of novel growth factors (14), or combinations of small molecule inhibitors to maintain HSCs ex vivo (15). This protocol is potential applications have highlighted the importance of collaboration between chemical biology and stem cell biology to overcome this barrier to human HSC expansion.

Reagents

Cell culture reagents and supplies:

1. Iscove's Modified Dulbecco's Medium (IMDM) (Gibco 12440053)
2. 100X Penicillin-Streptomycin-Glutamine (P/S/G) (Gibco 10378-016)

3. 100X Insulin-Transferrin-Selenium-ethanolamine (ITS-X) (Gibco 51500-056)

4. 100 mg/ml polyvinyl alcohol (PVA) (Sigma-Aldrich P8136) or polyvinyl caprolactam-polyvinyl accetate-polyethylene glycol graft copolymer (PCL-PVAc-PEG) (Soluplus®, BASF 50539897) dissolved in sterile DI water*.

5. 0.5 mM 740Y-P dissolved in sterile DI water (SCRUM Inc., Japan)

6. 10 μM butyzamide dissolved in DMSO (Shionogi & Co., Ltd., Japan)

7. 70 μM UM171 or 1 mM UM729 dissolved in DMSO (Stem Cell Technologies, #72914, #72332)

8. Sterile phosphate-buffered saline (PBS) (Gibco 10010-023)

9. CellBIND® 24-well Clear Multiple Well Plates (Corning 3337)

10. Falcon® Sterile 15 ml tube (Corning 352196)

*Note: See under protocol for how to dissolve PVA and Soluplus® in DI water.

Add 10 g of PVA or Soluplus® to a sterile flask and increase the solution to 100 ml with sterile water. The solution is dissolved by autoclaving at 121°C for 15 minutes and allowed to stand at room temperature for several days. Use after confirming that the color of the solution becomes transparent and there are no unmelted residues. Store the solution at room temperature.

Cell analysis reagents:

1. Trypan Blue solution (0.4%) (Gibco 15250-061)

2. APC-labeled anti-human CD34 antibody** (clone 581, BioLegend #343510)

3. BV421-labeled anti-human CD90 antibody** (clone 5E10, BioLegend #328122)

4. PE-labeled anti-human CD90 antibody** (clone 5E10, BioLegend # 329110)

5. PerCPCy5.5-labeled anti-human CD45RA antibody** (clone HI100, BioLegend #304121)

6. BV605-labeled anti-human CD201 antibody** (clone RCR-252, BD Bioscience #745335)

7. BV711-labeled anti-human CD41a antibody** (clone HIP8, BD Biosciences #740778)

8. PE-labeled anti-human CD49c (ITGA3) antibody** (clone C3 II.1, BD Biosciences #556025)

9. Propidium iodide (PI) solution (BioLegend #421301)

10. AlexaFluor488 Annexin-V (Invitrogen #A13201)

11. Falcon® 5ml Round Bottom Polyetyrene Test Tube with Cell Strainer Snap Cap (Corning 352235)

12. Falcon® Sterile 15 ml tube (Corning 352196)

**Note: Antibody concentration of should be individually titrated before use (antibody concentration will change between batches and vendors).

Human cord blood CD34 positive cells:

1. Human umbilical cord blood-derived (CB) $CD34^+$ cells were purchased from StemExpress (Folsom, CA, USA)

Equipment

Equipment:

1. Laminar-flow sterile tissue culture hood
2. Sterile pipettes and filter tips
3. Sterile electric pipet-aid and sterile strip-pipettes
4. Calibrated laboratory scales
5. Laboratory autoclave
6. Laboratory water-bath set to 37°C
7. Tissue culture incubator, humified and set to 37°C, 5% CO2, 20% O2
8. Fluorescence-activated cell analyzer (e.g., BD FACS AriaII, BD FACS Verse, BD LSR Fortessa)
9. Bench-top centrifuge set to 4°C

Procedure

Preparing PVA or Soluplus®-based HSC media:

1. Media should be prepared fresh for every use and media should be pre-warmed to 37°C before use.

2. Mix media reagents to make IMDM media supplemented with, 1X P/S/G, 1X ITSX, 1 mg/ml PVA or Soluplus®, 1 µM 740Y-P, 0.1 µM butyzamide and 70 nM UM171 or 1 µM UM729. Prepare enough for 1 ml per well. Note that PVA and Soluplus® solution are viscous and will need to be pipetted slowly. Mix media well by inversion before use.

3. Human CB $CD34^+$ cells are suspended in at 2 x 10^4 - 1 x 10^5 cells/ml. Transfer 1 ml media into desired 24-well CellBind plate wells.

Maintaining HSPC cultures:

1. Media changes must be performed every 2-3 days throughout the entire culture using pre-warmed and freshly prepared media. Inspect the cell cultures before performing each media change using a light microscope.

2. Collect all the cell culture media in the well into a 15 ml tube using a pipette.

3. Spin down at 440 g for 5 minutes at RT. After removing supernatants, add new media and resuspend in at 2 x 10^4 - 1 x 10^5 cells/ml. Seed 1 ml per well in 24-well plate.

4. After media changes, transfer back to the tissue culture incubator.

5. Cells cultures can be analyzed at any time point as described below. Alternatively, cell cultures can be used in in vivo transplantation assays (see other published protocols on the HSC transplantation assay) (4,7,8).

Analyzing cultured Human HSPC:

1. To count the cell cultures, gently pipette the cultures to dissociate attached cells. Transfer 10 ul of the culture to a tube. Mix 1:1 with Trypan Blue and count using a hemocytometer. Alternatively, an automated cell counter can be used.

2. Resuspend cells in the Stain Buffer (e.g. PBS+2% FCS) containing the antibodies against CD34, CD90, CD45RA, CD201 and CD49c. Stain cells at 4°C for 30 minutes, wash with at least 10 volumes of the Stain Buffer and spin down.

3. Resuspend cells in the Stain Buffer containing 1X PI, transfer to a FACS tube, and store at 4°C until analysis.

4. Analyze on a FACS machine/flow cytometer using unstained and single stained samples as controls.

Troubleshooting

Lack of cells after culture: Check that your starting population has CD34-highly positive- cell frequencies by antibody staining and flow cytometry. Make sure to gently perform media changes and check that you are not removing all cells during media changes.

Low cell viability after culture: Always prepare your media fresh, pre-warmed, and well-mixed. Confirm the activity of your chemicals (740Y-P, butyzamide and UM171/UM729). Check that your starting population has non-apoptotic $CD34^+$ cells by Annexin-V binding assay (according to manufacturer's instructions). Service your tissue culture incubator. Consider initiating the cultures at higher or lower densities.

Time Taken

Preparing PVA or Soluplus®-based media: 15 minutes.

Maintaining HSPC cultures: 15-30 minutes every 2-3 days, depending on number of cells.

Analyzing HSPC cultures: 1-4 hours, depending on the number of samples

Total ex vivo HSPC culture: Usually 2-4 weeks but depends on downstream application.

Anticipated Results

Please see Associated Publication.

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Acknowledgements

We thank M. Watanabe, Y. Yamazaki, Y. Ishii, M. Hayashi, R Hirochika, M. Kikuchi and Organization for Open Facility Initiatives, University of Tsukuba for excellent technical support, and Y. Niitsu, M. Kawakatsu, T.Kajiura, K. Kolter and F. Guth for providing polymers. This research was funded by JSPS KAKENHI Grant-in-Aid for Scientific Research (JP20H03707; JP20H05025; JP20K17407) and the Japan Agency for Medical Research and Development (AMED) (21bm0404077h0001; 21bm0704055h0002).