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Abstract

Purpose:

In recent years, human hematopoietic stem cells (HSC) have become a valuable resource for the repopulation of the hematopoietic system following high-dose chemotherapy. HSC can be purified from several sources, most prominently bone marrow, peripheral blood and umbilical cord blood. In many cases, the cell number obtained after purification is not sufficient for transplant and must be increased utilizing *ex vivo* expansion. A successful expansion must provide enough fully functional material to combat neutropenia and thrombocytopenia in the early stages post-transplant and lead to long-term engraftment of the patient. To this end, we have co-developed a serum-free medium (marketed as Hematopoietic Stem Cell Growth and Expansion Medium [HSC GEM], Stemgenix, Amherst, NY and as Stemline™, Sigma-Aldrich, St. Louis, MO) for the optimal expansion of HSC. In this medium, human serum albumin is the only animal-origin component present.

Methods:

Our product was compared with other commercially available serum-free expansion media for the ability to expand total nucleated cells (TNC) and CD34⁺ cells in a 24-well microplate culture system and in a 2-step, clinical-scale protocol using Teflon® culture bags. Clinical scale cultures were also assayed for presence of committed progenitors (GM-CFC) and primitive, high proliferative potential progenitors (HPP-CFC).

Results:

In the microplate culture system, use of HSC GEM/Stemline™, when compared to other serum-free media, provides a significantly increased expansion of TNC from cultures of CD34⁺ cord blood cells, bone marrow and mobilized peripheral blood. Flow cytometric data indicates increased specific expansion of CD34⁺ cells and clinical scale data also supports the overall greater expansion of TNC and CD34⁺ cells in HSC GEM/Stemline™, as well as the expansion of both committed and primitive progenitor compartments.

Conclusions:

Results indicate that HSC GEM/Stemline™ provides a significant benefit over other commercially available serum-free formulations, such as X-VIVO 15™ and StemSpan H2000™, for the expansion of TNC, committed progenitor and primitive progenitor compartments. This suggests that in the clinical stem cell transplant setting, HSC GEM/Stemline™ may provide significant benefit toward reducing time-to-engraftment and may also result in the reduction in frequency and/or severity of neutropenia and thrombocytopenia.

Introduction

The use of human hematopoietic stem cells (HSC) as a source of cellular reconstitution following high-dose chemotherapy is now a common therapeutic modality for the treatment of malignancy. These HSCs can be purified from several sources, including umbilical cord blood (CB), bone marrow or mobilized peripheral blood. CB grafts are especially beneficial, because clinical data indicates that there is a reduced incidence and severity of graft vs. host disease (GVHD), apparently due to the decreased alloreactive potential of fetal lymphocytes present in CB.¹ However, CB products contain low numbers of total cells and progenitor cells, which have limited the use of CB primarily to smaller pediatric patients. In order to obtain optimal numbers of HSCs for transplantation in adults, *ex vivo* expansion has been explored to ensure successful engraftment and minimize the short-term effects of neutropenia and thrombocytopenia. A medium/cytokine combination that could provide high levels of expansion of long-term, high proliferative potential engrafting cells as well as committed progenitors could serve to enhance the therapeutic outcome achieved with HSC transplant. To this end, we have developed a serum-free medium, HSC GEM™ (Stemgenix, Amherst, NY)/Stemline™ Hematopoietic Stem Cell Expansion Medium (Sigma-Aldrich Corp., St. Louis, MO) for the optimal expansion of HSC. In this medium, human serum albumin is the only animal-origin component present. This medium has also been evaluated in the 3 primary cell sources, in both the traditional cell culture well plate format and in a clinical-scale expansion format, with both generating promising results for *ex vivo* expansion and functionality.

Materials & Methods

Cell Preparation

For research-scale experiments, cryopreserved, human CD34⁺ cells were obtained from independent suppliers (Poietics/BioWhittaker; Walkersville, Maryland) (AllCells, LLC; Berkeley, California) and were handled in a manner consistent with the manufacturer's instructions with regard to storage and reconstitution. Cells were counted using either a hemocytometer or Guava Personal Cytometer (Guava Technologies; Hayward, California) to determine cell density and viability. CD34⁺ cells used in clinical-scale expansions were derived with informed consent from umbilical placental veins according to the protocol described by McNiece et al.³ or purchased from Poietics/BioWhittaker (Walkersville, Maryland).

Serum Free Expansion Medium Preparation

HSC GEM (Stemgenix; Amherst, New York)/Stemline (Sigma-Aldrich; St. Louis, Missouri) Hematopoietic Stem Cell Expansion Medium, X-VIVO 15 (BioWhittaker; Walkersville, Maryland), HPGM (BioWhittaker; Walkersville, Maryland), SCGM (Cellgenix, Gaithersburg, Maryland), QBSF-60 (Quality Biological, Gaithersburg, Maryland), HemaPro (Celox, St. Paul, Minnesota), StemPro-34 (Life Technologies; Grand Island, New York) and StemSpan H2000/StemSpan SFEM (StemCell Technologies; Vancouver, British Columbia) were purchased fresh, aliquoted and stored according to the manufacturer's recommendations. For each experiment, a 10 ml volume of each expansion medium was warmed to 25 °C. One ml triplicate aliquots of each medium were established in 24 well culture plates (Corning/Costar; Corning, New York) to which SCF, TPO and G-CSF (Sigma-Aldrich; St. Louis, Missouri) were added to a final concentration of 100 ng/ml. Sterile PBS (Sigma-Aldrich; St. Louis, Missouri) was added to unused wells to maintain humidity. Plates were incubated at 37 °C and 5% CO₂ for 1/2 hour prior to the addition of the revived CD34⁺ cells. Viable recovered CD34⁺ cells were added to each well at 1.0 x 10⁴ cells/ml and allowed to proliferate in a humidified incubator at 37 °C and 5% CO₂ for 7 or 14 days. Following the appropriate incubation period, 24 well plates were removed for counting.

Flow Cytometry

Pooled cells from each sample were washed with PBS and centrifuged at 1500 g (3200 rpm) for 3 minutes. After removing all but 1 ml of the supernatant, cells were resuspended and 200 µg normal mouse IgG (Caltag, Burlingame, California) was added to the cell suspension for 15 minutes on ice to prevent non-specific binding of reagents to Fc receptors. Following the incubation period, 50 µl blocked cells were added to 20 µl each FITC conjugated reagent (APC anti-CD34, FITC anti-CD15, FITC anti-CD41 and isotype control (Becton Dickinson; San Jose, California)). Again, cells were incubated in the dark for 15 minutes on ice after light vortexing. Cells were then washed and fixed with 200 µl 2% formaldehyde. Refrigerated cells were analyzed in the laboratory of Dr. Carleton Stewart at Roswell Park Cancer Institute using a Becton Dickinson FACSort flow cytometer.

Clinical Scale Expansion

The study was then expanded at the University of Colorado Bone Marrow Transplant Center to a 2-step, clinical-scale protocol using Teflon® culture bags (American Fluoroseal, Inc.; Gaithersburg, Maryland). Cultures were assayed for TNC, committed progenitors (GM-CFC) and primitive, high proliferative potential progenitors (HPP-CFC). For clinical-scale studies, CB CD34⁺ cells were harvested as described by McNiece et al.³ and cultured for 7 days in 100 ml Teflon® culture bags containing 50 ml of each culture medium plus cytokine concentrations as previously described. Cells were harvested from these bags and a 5 ml aliquot was transferred to a second 100 ml Teflon® bag containing 45 ml of each selected medium plus cytokines and cultured for an additional 7-day culture period. At the end of the culture protocol, cells were harvested and TNC counted by hemocytometer. Methylcellulose assay for committed (GM-CFC) and primitive progenitors (HPP-CFC) were performed as described below.

Methylcellulose Assay

GM-CFC and HPP-CFC populations were enumerated by CFU assay. Briefly, 1 x 10⁴ cells of each expanded population were added to 3 ml of complete methylcellulose without erythropoietin (Stemgenix; Amherst, New York) and plated in triplicate into Falcon cell culture dishes (Becton Dickinson; San Jose, California). Three 35 mm culture dishes plus one 35 mm dish containing sterile PBS were incubated in a 100 mm Petri dish at 100% humidity, 37 °C and 5% CO₂. Plates were scored using a dissecting microscope at 20X magnification on day 14 for GM-CFC and day 28 for HPP-CFC. Final CFC numbers were generated by multiplying the raw CFC count by the total number of expanded cells and then dividing by the methylcellulose plating density.

Results

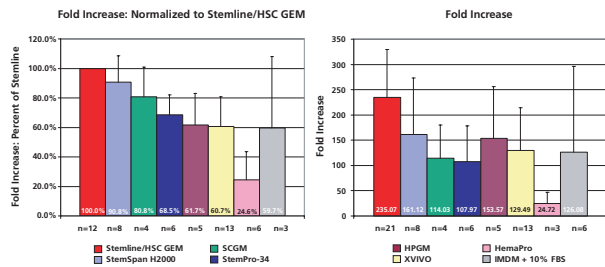


Figure 1 Total nucleated cell expansion from CD34⁺ umbilical cord blood cells. Cells were seeded in triplicate at 10,000 cells per well in 24-well tissue culture plates containing 1 ml of the appropriate expansion medium per well. After a 14 day incubation, cells were counted and the fold increase was determined (cells_{final} ÷ cells_{initial}). In umbilical cord blood, Stemline/HSC GEM outperforms both serum-containing medium and all serum-free media on the market.

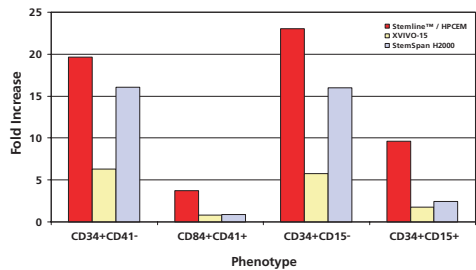


Figure 2 Flow cytometric analysis of CD34⁺ cord blood cells expanded in 24-well tissue culture plates.

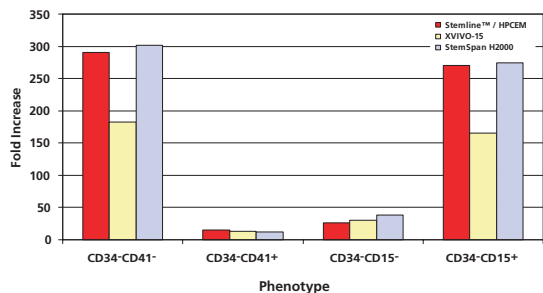


Figure 3 Flow cytometric analysis of CD34⁺ cord blood cells expanded in 24-well tissue culture plates.

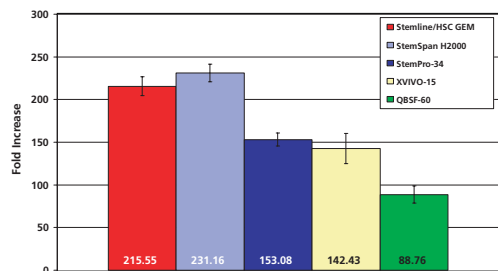


Figure 4 Expansion of total nucleated cells from CD34⁺ bone marrow (One representative experiment). Cells were seeded in triplicate as in Figure 1. After a 14 day incubation, cells were counted and the fold increase was determined. Expansion of bone marrow-derived cells was not as robust as expansion of umbilical cord blood cells. Stemline/HSC GEM was either superior or equivalent to the serum-free formulations.

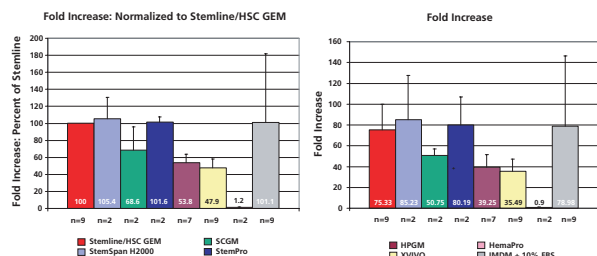


Figure 5 Expansion of CD34⁺ mobilized peripheral blood cells. Cells were plated as in Figure 1. After a 14 day incubation, cells were counted and the fold increase was calculated. Expansion of total nucleated cells from CD34⁺ mobilized peripheral blood also proved Stemline/HSC GEM to be either superior or equivalent to the current available formulations.

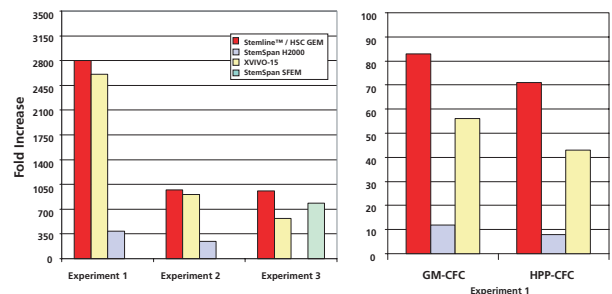


Figure 6 Two-step, clinical-scale expansion of CD34⁺ cord blood cells in Teflon culture bags. Cells were seeded with the appropriate cytokines in 50 ml of expansion medium in 100 ml culture bags. After a 7 day incubation, cells were harvested and passed into a second 100 ml culture bag. The second bag was incubated for an additional 7 days. Cells were assayed for expansion of total nucleated cells and functionality via CFU assay. Stemline/HSC GEM was superior to the serum-free formulations tested in this clinical application.

Conclusion

Bench-Scale:

- For expansion of CD34⁺ cells derived from cord blood, Stemline/HSC GEM provides a higher TNC fold increase than other commercially available serum-free medium formulations, when using identical cytokine combinations.
- When culturing bone marrow-derived CD34⁺ cells, Stemline/HSC GEM provides an equivalent TNC fold increase to StemSpan H2000, and surpasses the expansion potential of all other commercial products tested.
- Stemline/HSC GEM provides an equivalent TNC fold increase to StemSpan H2000, StemPro-34 and serum control when using CD34⁺ cells isolated from mobilized peripheral blood and surpasses the expansion potential of other tested commercially available products.
- Flow cytometric analysis of the research scale expansion products derived from cord blood reveals that the Stemline/HSC GEM formulation generates a significant expansion of committed (CD34⁻ / CD15⁺, CD41⁺) and early (CD34⁺ / CD15⁻, CD41⁻) progenitors.

Clinical-Scale:

- Although StemSpan H2000 demonstrates a reasonable expansion of TNC and late progenitors in the bench-scale studies and X-VIVO 15 exhibits a decreased potential, clinical-scale experiments show a different result.
- Stemline/HSC GEM, X-VIVO 15 and StemSpan SFEM (single experiment) share similar TNC proliferative capacities, however, Stemline/HSC GEM provides a larger expansion of early (165%) and committed (148%) progenitors than X-VIVO 15.
- StemSpan H2000 does not perform well in this clinical expansion format.

References

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