Cell Culture

Serum-Free Expansion of CD34+ Umbilical Cord Blood Using Stemline™ Hematopoietic Stem Cell Expansion Medium

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Introduction

Human hematopoietic stem cells (HSC) have become a valuable resource for the repopulation of the hematopoietic system following high-dose chemotherapy. HSC are purified from three primary sources: bone marrow, peripheral blood, and umbilical cord blood (CB). CB grafts are gaining favor, since clinical data indicates there is a reduced incidence and severity of graft versus host disease, attributed to the decreased alloreactive potential of fetal lymphocytes present in CB.[1] However, CB cell isolates contain low numbers of total cells and progenitor cells, which have limited the use of CB grafts to smaller pediatric patients. In order to obtain optimal numbers of HSC for transplantation in adults, *ex vivo* expansion has been explored to ensure not only successful engraftment, but also minimize the short-term effects of neutropenia and thrombocytopenia.[2] A medium/cytokine combination that provides 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, animal protein-free medium, Stemline™ Hematopoietic Stem Cell Expansion Medium / HSC GEM™ (Sigma-Aldrich Corp., St. Louis, MO / Stemgenix, Amherst, NY) for the optimal expansion of HSC. This medium has been evaluated in the three primary cell sources, in both the traditional cell culture well plate format and in a clinical-scale expansion format. Results from both experimental formats demonstrate superior expansion and functionality. It is anticipated that the use of Stemline™ Hematopoietic Stem Cell Expansion Medium / HSC GEM™ combined with an optimal cytokine regimen *for ex vivo* expansion, would lead to a significantly reduced time for successful engraftment in cancer patients.

Materials & Methods

All materials were supplied by Sigma-Aldrich Corporation (St. Louis, MO) unless otherwise stated.

Cell Source and Handling

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

Bench-Scale Expansion Protocol

Stemline™ Hematopoietic Stem Cell Expansion Medium (marketed as HSC GEM™ by Stemgenix), and all competitor media 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 placed in the wells of 24-well culture plates (Corning/Costar; Corning, New York) to which stem cell factor, thrombopoietin and granulocyte-colony stimulating factor were each added to a final concentration of 100 ng/ml. Sterile PBS was added to unused wells to maintain humidity. Plates were incubated at 37 °C, 5 % CO_2 for 1/2 hour prior to the addition of the viable recovered CB CD34+ cells. Revived CB CD34+ cells were added to each well at 1.0×10^4 cells/ml and allowed to proliferate in a humidified incubator at 37 °C, 5 % CO_2 for 14 days. Following the appropriate incubation period, plates were removed for counting.

Flow Cytometry

Pooled cells from each sample were washed with PBS and centrifuged at 1500 x 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 and incubated for 15 minutes on ice to prevent non-specific binding of reagents to F_C receptors. Following the incubation period, 50 µl of blocked cells were added to 20 µl of each fluorophore 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, fixed with 200 µl of 2 % formaldehyde, and stored at 4 °C. FACS analysis was performed in the laboratory of Dr. Carleton Stewart at Roswell Park Cancer Institute using a Becton Dickinson FACSort flow cytometer.

Clinical-Scale Expansion

At the University of Colorado Bone Marrow Transplant Center and Stemgenix, a 2-step, clinical-scale protocol using Teflon® culture bags (American Fluoroseal, Inc.; Gaithersburg, Maryland) was employed. Cultures were assayed for total nucleated cells (TNC), committed progenitors (granulocyte-macrophage-colony forming cells, GM-CFC) and primitive progenitors (high proliferative potential colony forming cells, 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 were counted by hemocytometer. Methylcellulose assays for committed (GM-CFC) and primitive progenitors (HPP-CFC) were performed as described in the following protocol.

Methylcellulose Assay

GM-CFC and HPP-CFC populations were enumerated by colony-forming unit (CFU) assay. Briefly, 1 x 10^4 cells of each expanded population (from the clinical-scale expansion) were added to 3 ml of complete methylcellulose without erythropoietin (Stemgenix, Amherst, New York) and plated in triplicate into Falcon 35-mm cell culture dishes (Becton Dickinson; San Jose, California). The experimental dishes plus one dish containing sterile PBS were incubated in a 100-mm Petri dish at 100 % humidity, 37 °C and 5 % CO_2. Colonies were scored morphologically 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 and Discussion

Use of Stemline™ / HSC GEM™ at the bench-scale, provides increased expansion of TNC from CB $CD34^+$ cells. Figure 1 shows a comparison of Stemline™ / HSC GEM™ to six of the leading serum-free formulations on the market and a serum-containing formulation. CB $CD34^+$ cells from a variety of donors were expanded and for each assay the test medium was normalized to the fold expansion of Stemline™ / HSC GEM™. On average, Stemline™ / HSC GEM™ outperforms serum-containing medium and all of the serum-free formulations commonly used in the clinical setting. The increased number of TNC generated in this medium represents a significant improvement over other commercially available formulations.

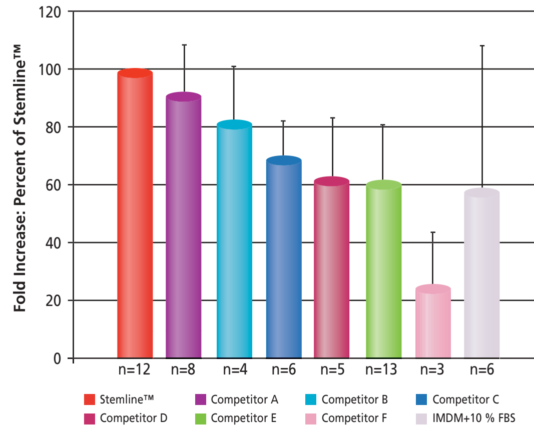

Figure 1. Total nucleated cell expansion from CB $CD34^+$ cells. *Cells were seeded in triplicate at 10,000 cells per well in 24-well tissue culture plates (bench-scale) containing 1 ml of the appropriate expansion medium per well. After a 14-day incubation, cells were counted and the fold increase was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

Flow cytometric analysis of the bench-scale expansion products reveals that the Stemline™ / HSC GEM™ formulation generates a significant expansion of committed ($CD34^-$ / $CD15^+$, CD 41^+) and early ($CD34^+$ / $CD15^-$, CD 41^-) progenitors. In order for the expanded cells to be useful in the clinic, they must retain the capacity to differentiate into all of the hematopoietic lineages. Figure 2 shows flow cytometric plots comparing Stemline™ / HSC GEM™ to the two major competitors. Cells were labeled with antibodies against CD34, CD41 and CD15 to determine the presence of those cell populations. Figure 3 shows this data as a graphical representation of the expanded CB $CD34^+$ cells. Stemline™ / HSC GEM™ consistently generates more $CD34^+$ progenitors than either of the competitor formulations. Stemline™ / HSC GEM™ also shows an enhanced expansion of committed cells as well ($CD34^-$/$CD15^+$ or $CD34^-$/$CD41^+$, data not shown).

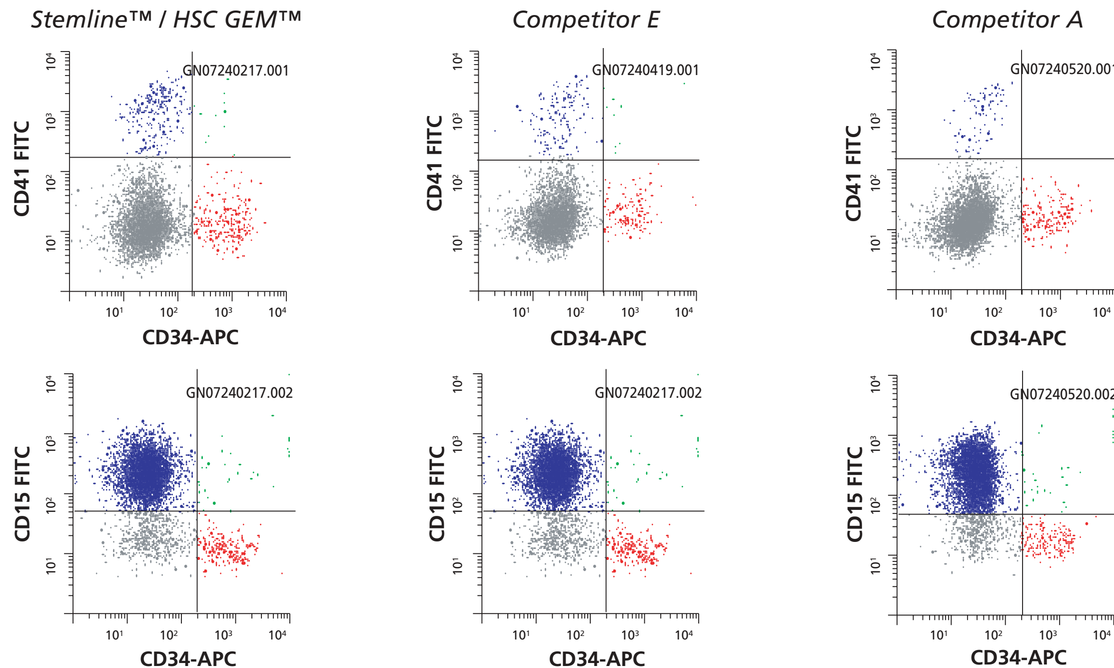

Figure 2. Flow cytometric analysis of a bench-scale expansion of CB $CD34^+$ cells. *Dot plots from the flow cytometer show an increased expansion of $CD34^+$ cells in Stemline™ when compared to the two competitors. There is also an increase in the expansion of $CD34^-$ committed cells in Stemline™ / HSC GEM™.*

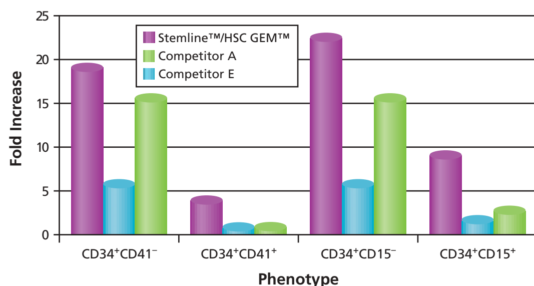

Figure 3. Flow cytometric analysis of a bench-scale expansion of CB CD34+ cells. *When represented graphically, the dot plots from the flow cytometer show a significant increase in the expansion of all CD34+ cells in Stemline™ / HSC GEM™ when compared to the two competitors.*

In clinical-scale protocols, Stemline™ / HSC GEM™ produces an expanded CB CD34+ culture with a greater capacity for generation of early and late progenitors. TNC expansion levels of Stemline™ / HSC GEM™ versus the leading competitors were assayed in three separate CB donors (Figure 4A). Stemline™ / HSC GEM™ consistently outperforms the competitors for the expansion of TNC in the clinical-scale format. As a test of functionality post-expansion, CFU assays can be used to indicate whether or not the cells are actually capable of differentiating into the appropriate range of cell lineages. Stemline™ / HSC GEM™ showed significantly greater expansion of both early (HPP-CFC) and late (GM-CFC) progenitor populations.

Conclusions

For expansion of CD34+ cells derived from cord blood in both bench-scale and clinical-scale applications, Stemline™ / HSC GEM™ provides a higher TNC fold increase than other commercially available serum-free medium formulations, when using identical cytokine combinations. In bench-scale experiments, Stemline™ / HSC GEM™ also provides for high levels of expansion for cells derived from either bone marrow or mobilized peripheral blood (data not shown). Flow cytometric analysis of the bench-scale expansion products derived from cord blood reveals that the Stemline™ formulation generates a significant expansion of committed (CD34− / CD15+, CD 41+) and early (CD34+ / CD15−, CD 41−) progenitors. In clinical-scale experiments, Stemline™ / HSC GEM™ not only exhibits high TNC proliferative capacities, but a greater expansion of early and committed progenitors than competitor product.

The greater overall expansion of total nucleated cells, along with the increased specific expansion of early progenitor populations identifies Stemline™ / HSC GEM™ as the superior serum-free product for hematopoietic research. Additionally, the ability to produce this medium in a state-of-the-art cGMP facility, with an available Device Master File (DMF) makes Stemline™ / HSC GEM™ the product of choice for clinical applications.

References

1. Noort, W.A. and Falkenberg, J.H.F., Hematopoietic content of cord blood. In: Cohen, S.B.A., Gluckman, E., et al (eds.), Cord blood characteristics: Role in Stem Cell Transplantation. London: Martin-Dunitz, pp. 13–37, (2000).

2. McNiece, I.K., et al. *Ex vivo* expanded cord blood cells provide rapid engraftment in fetal sheep but lack long term engrafting potential. Blood **98,** 476A, (2001).

3. McNiece, I.K., et al. Increased expansion and differentiation of cord blood products using a two-step expansion culture. Experimental Hematology **28,** 1181-1186, (2000).

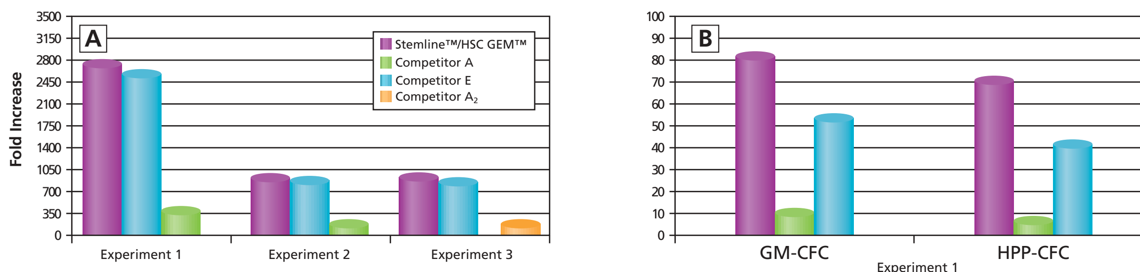

Figure 4. Two-step, clinical-scale expansion of CB CD34+ cells in Teflon® culture bags. *Cells were seeded with the appropriate cytokines in 50 ml of expansion medium in 100-ml culture bags. After a 7-day incubation, cells were harvested, passed into a second 100-ml culture bag, incubated for an additional 7 days and then assayed for fold expansion* ***(A)*** *and generation of both late and early progenitors via a CFU assay* ***(B).*** *Stemline™ / HSC GEM™ was superior to the serum-free formulations tested for the expansion of TNC, GM-CFC (late progenitors) and HPP-CFC (early progenitors). Note: A_2 is a modification of A, containing BSA rather than HSA.*

About the Authors

Dan Allison, Ph.D., Stacy Leugers, B.S., and Brad Fuhr, B.S. are Scientists and Laurie Donahue, Ph.D. is Manager of Clinical Cell Culture at Sigma-Aldrich in St. Louis, MO. Frank Swartzwelder, Ph.D. and Joseph Tario, Jr. are President/CSO and Associate Scientist, respectively, at Stemgenix in Amherst, New York. Jenny Harrington, B.S. and Ian McNiece, Ph.D., are Professional Research Associate and Professor of Medicine, respectively, in the Bone Marrow Transplant Program at the University of Colorado Health Sciences Center in Denver, CO.

ORDERING INFORMATION

Product Code	Product Description	Unit
S 0189	Stemline™ Hematopoietic Stem Cell	500 mL
	Expansion Medium Liquid	6 x 500 mL

SUGGESTED LITERATURE

H 7911	Hematopoietic Stem Cell Protocols (2002)	1 each
S 8066	Stem Cell Biology (2001)	1 each