

Ex Vivo Expansion of CD34⁺ Cells in Stemline™ II Hematopoietic Stem Cell Expansion Medium Generates a Large Population of Functional Early and Late Progenitor Cells

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Abstract

Hematopoietic stem cell replacement therapy is an area of research lacking an optimal culture system that allows for the *ex vivo* expansion of CD34⁺ cells for transplant. The necessity for expansion is due to the lack of sufficient material from umbilical cord blood (UCB), the preferred source of CD34⁺ cells. UCB transplantations are preferred because fewer patients suffer from graft-versus-host disease compared to transplantations using CD34⁺ cells isolated from mobilized peripheral blood or bone marrow. In addition to the source of the starting material, the final composition of the expanded cells is also very important to a successful transplant. The expanded transplantation material must contain both early and late progenitor cells to ensure the long-term engraftment that is required in patients with various genetic disorders and those that have gone through ablative, high dose chemotherapy.

Stemline™ II Hematopoietic Stem Cell Expansion Medium was developed to facilitate the development of this replacement therapy. Culturing CD34⁺ cells in Stemline™ II consistently yields higher levels of expansion than seen with other commercially available serum-free media. Analysis of the expanded material for lineage indicating markers by flow cytometry demonstrates that the material contains the required early and late progenitors. Furthermore, NOD/SCID studies confirm that the expanded cells are capable of long-term engraftment and are therefore functional. Together, this data supports the conclusion that culturing CD34⁺ cells in Stemline™ II Hematopoietic Stem Cell Expansion Medium is a key step in developing the optimal culture system.

Introduction

Hematopoietic stem cells (HSC) have the ability to repopulate the hematopoietic system by differentiating into all of the necessary erythroid, lymphoid, and myeloid lineages. Due to this rare ability, HSCs are used as therapeutic agents in the treatment of malignant and benign diseases of the blood forming and immune systems. There have been many advances in the area of clinical HSC research, but the availability of suitable cells for transplantation still remains a major limiting factor.

HSCs can be isolated from three different sources: umbilical cord blood (CB), bone marrow, and mobilized peripheral blood. CB is currently the preferred source because it has been shown to have a lower risk of graft-versus-host disease (GVHD), presumably due to its immunological naivete. However, because the volume of CB is limited, each umbilical cord has only enough cells to successfully transplant a small child. In order to transplant an adult, the HSCs from CB must be expanded *ex vivo*. The expansion must be performed in a manner to ensure that the HSCs not only differentiate along appropriate hematopoietic lineages, but also self-renew, leaving undifferentiated stem cells in the expanded culture. The differentiated cells will allow for short-term engraftment that will reduce the effects of neutropenia and thrombocytopenia in the patient. The undifferentiated cells will allow for long-term engraftment that will establish a new, permanent hematopoietic system for the patient. In order to expand these very specific cell types in the absence of potentially adventitious agents such as fetal bovine serum, an optimized serum-free medium and cytokine cocktail are needed.

To this end, Stemline™ Hematopoietic Stem Cell Expansion Media were developed for the expansion of HSCs. They are serum-free media that allow for expansion of both differentiated and undifferentiated HSCs. Stemline™ and Stemline™ II are both able to expand HSCs from CB, bone marrow, and mobilized peripheral blood. In bench-scale and clinical-scale expansions, both media have shown promising results in expanding a mixed population of cells that remain fully functional. The original medium, Stemline™, expands CD34⁺ cells better than or equal to other commercially available serum-free HSC media. Stemline™ II is a newer version of the medium that has an increased expansion potential for CD34⁺ cells.

Materials & Methods

Cell Preparation

For all experiments, cryopreserved, human CD34⁺ cells were obtained from independent suppliers (Stemgenix, Amherst, New York; AICells, LLC, Berkeley, California; Cambrex, Walkersville, Maryland) 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.

Serum-Free Expansion Medium Preparation and Bench-Scale Expansion

All materials are from Sigma-Aldrich unless otherwise noted. Stemline™ Hematopoietic Stem Cell Expansion Media, IMDM, X-VIVO 15 (Cambrex, Walkersville, Maryland), HPGM (Cambrex, Walkersville, Maryland), QBSF-60 (Quality Biological, Gaithersburg, Maryland), StemPro-34 (Invitrogen, Carlsbad, California), and StemSpan H3000 (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 of each medium was pipetted in triplicate in 24-well culture plates (Corning/Costar, Corning, New York) to which SCF, TPO and G-CSF were added to a final concentration of 100 ng/ml each. Sterile PBS was added to unused wells to maintain humidity. Plates were incubated at 37 °C and 5% CO₂ for 15 minutes 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 10 days. Following the incubation period, the expanded total nucleated cells were counted.

Flow Cytometry

The direct determination of the absolute count of CD38⁺ and CD38⁻ cells was assessed utilizing either the Immunotech Stem Kit CD34⁺ Hematopoietic Progenitor Cell (HPC) Enumeration kit (Beckman-Coulter, Fullerton, CA), CD38-PE, CD34-ECD, and CD45-FITC antibodies or CD34-FITC and CD38-PE (BD Biosciences, San Jose, CA). The processed samples were identified and enumerated using either Coulter's flow cytometer (EPICS XL-MCL) or BD's FACSscan.

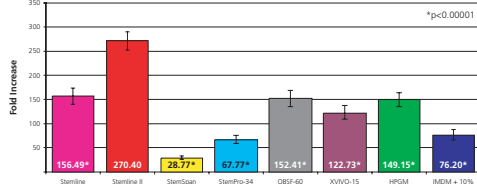
Clinical-Scale Expansion

Two-step, clinical-scale assays (McNiece, et al., *Experimental Hematology* 2000, 28: 1181-1186) using Teflon™ culture bags (American FluoroSeal, Inc., Gaithersburg, Maryland) were set up. For clinical-scale studies, CB CD34⁺ cells were cultured for 7 days in 100 ml Teflon™ culture bags containing 50 ml of each culture medium plus SCF, TPO and G-CSF at a final concentration of 100 ng/ml each and FLT-3 at a final concentration of 50 ng/ml. Cells were harvested from these bags and a 10 ml aliquot was transferred to a second 100 ml Teflon™ bag containing 90 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, counted by hemocytometer, viability tested, and assayed for functional hematopoietic activity by flow cytometric analysis, colony forming unit (CFU) assay, and NOD/SCID assay.

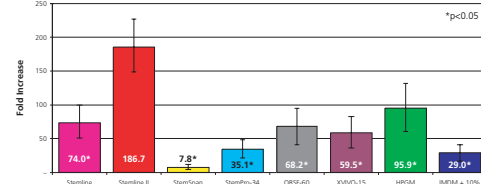
NOD/SCID Studies

Immunodeficient NOD/SCID mice were used as recipients of transplanted human cord blood cells expanded at clinical-scale as described above. NOD/SCID mice were lightly irradiated (3.0 Gy) 3-6 hrs prior to infusion of human cells by injection intravenously into the retro-orbital plexus, as described in detail by Szilvassy et al. (in Hematopoietic Stem Cell Protocols, Klug and Jordan eds., Humana Press, 2001, pp147-167). The mice prior to and throughout the treatment period were maintained under pathogen barrier conditions and their drinking water was supplemented with antibiotics. Depending on the experiment, between 500,000 and 6 million cells were transplanted. At various times after transplant, blood was obtained from each recipient by retro-orbital blood sampling. Human cells were distinguished by immunofluorescence and flow cytometry using CD45 antibody (Becton-Dickinson, Franklin Lakes, New Jersey). CD34 was used to distinguish progenitor cells and representation of human cells in the lymphocyte lineage was determined using CD19 and CD20; human myeloid cells were identified by CD15 and CD66b. The same panel of antibodies was used in the analysis of bone marrow at the termination of the experiment, or in preparation for injection into secondary recipients to verify the presence of self-renewing human stem cells in the primary recipients, and ultimately in the cord blood cells expanded in both Stemline™ culture media.

Fold Increase of Total Nucleated Cells from CD34⁺ Cord Blood (Mean ± S.E.M.; n = 15 donors)



Fold Increase of Total Nucleated Cells from CD34⁺ Mobilized Peripheral Blood (Mean ± S.E.M.; n = 7 donors)



Viable Cell Increase for CD34⁺ Cord Blood Cells in Stemline™ II Medium-Clinical Scale

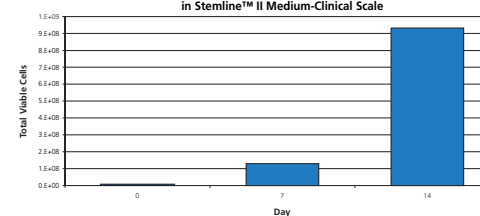
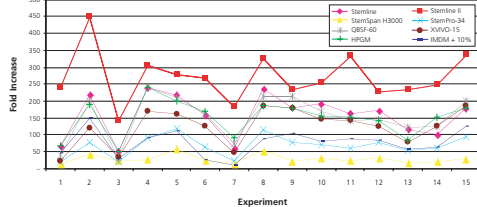


Figure 6: Clinical-scale expansion of CD34⁺ cells from cord blood in Stemline™ II medium. Another set of clinical-scale experiments was initiated to compare the functionality of unexpanded CD34⁺ cells to CD34⁺ cells expanded in Stemline™ II. Starting with 2.46k viable cells, the cells were cultured according to the two-step clinical-scale expansion protocol and achieved a 385 fold increase in viable cells. The starting material and the expanded material were analyzed by flow cytometry and CFU assay before being injected into NOD/SCID mice. This will determine whether or not functionality is being compromised by expanding the CD34⁺ cells. Assays are still in progress.

Fold Increase of Total Nucleated Cells from CD34⁺ Cord Blood (n = 15 donors)



% of Total Cells Expressing CD34 Relative to Stemline™, with Corresponding CD38 Expression (n = 2 Mobilized Peripheral Blood donors; average ± S.E.M.)

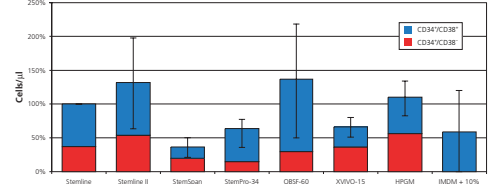
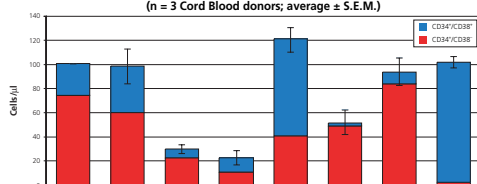


Figure 3: Bench-scale expansion of CD34⁺ cells from mobilized peripheral blood in Stemline™ and Stemline™ II. Assays were set up as in Figure 1. The cells were counted on day 14 and the fold increase was determined by (cells_{day 14}/cells_{day 0}). In mobilized peripheral blood, both Stemline™ products consistently exhibit high levels of expansion of total nucleated cells (n = 7, p<0.05). Flow cytometry on the expanded cells reveals that Stemline™ II also expands a large number of CD34⁺ stem cells (both CD38⁺ and CD38⁻). Cells/yL is normalized to the average number of cells/yL in Stemline™ + S.E.M. (n = 2 mobilized peripheral blood donors).

% of Total Cells Expressing CD34 Relative to Stemline™, with Corresponding CD38 Expression (n = 3 Cord Blood donors; average ± S.E.M.)



Average Viable Cell Increase for CD34⁺ Cord Blood Cells in Stemline™ Media-Clinical Scale (n = 2)

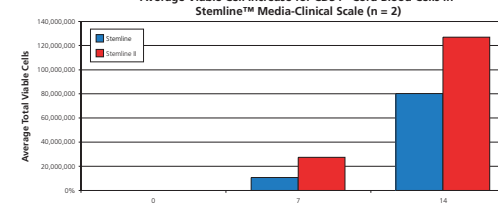
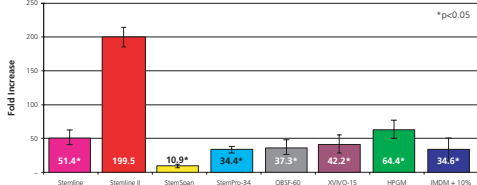
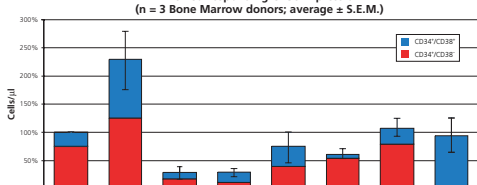


Figure 4: Comparison of Stemline™ to Stemline™ II in a clinical-scale expansion of CD34⁺ cells from cord blood. A two-step clinical-scale expansion was performed to compare cell growth in Stemline™ and Stemline™ II. Briefly, approximately 3.56k cells were seeded into 100 ml bags and incubated for 7 days. On day 7, a portion of the expanded cells was inoculated into a fresh 100 ml bag for an additional 7 days. Both media demonstrate increased potential for expanding CD34⁺ cells from cord blood, supporting excellent growth and high viability (>80%). On average, the culture in Stemline™ reached a 238 fold increase in viable cells while the Stemline™ II culture achieved a 377 fold increase.

Fold Increase of Total Nucleated Cells from CD34⁺ Bone Marrow (Mean ± S.E.M.; n = 5 donors)



% of Total Cells Expressing CD34 Relative to Stemline™, with Corresponding CD38 Expression (n = 3 Bone Marrow donors; average ± S.E.M.)



Flow Cytometric Analysis of CD34⁺ Cord Blood Cells from the Clinical-Scale Expansion

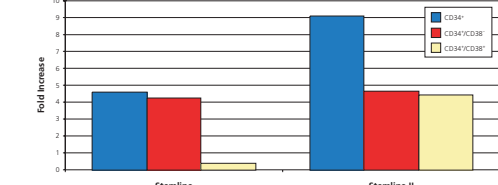


Figure 5: Flow cytometric analysis on CD34⁺ cord blood cells from clinical-scale expansion. After the two-step expansions were complete, a sample of cells from the Stemline™ and Stemline™ II cultures was analyzed by flow cytometry for expression of CD34 and CD38. The majority of the CD34⁺ cells expanded in Stemline™ remained undifferentiated, early progenitors (CD34⁺CD38⁻), while cells expanded in Stemline™ II contained both early (34⁺CD38⁻) and late progenitor (34⁺CD38⁺) phenotypes. Both media expand high levels of early progenitors, which is important for long-term engraftment. Stemline™ II also expands high levels of the late progenitors required for early engraftment and amelioration of the post-transplant nadir in mature myeloid cells.

	Stemline™			Stemline™ II			
	Injected Cells	600,000	1,800,000	5,400,000	600,000	1,800,000	5,400,000
Survival Rate	5/10	3/10	3/7	7/10	6/10	6/7	86%
Average % CD45 ⁺	0.064 ± 0.061	0.017 ± 0.006	0.143 ± 0.081	0.036 ± 0.013	0.018 ± 0.019	0.108 ± 0.162	
Average % CD45 ⁺ /CD34 ⁺	0.000 ± 0.000	0.003 ± 0.006	0.007 ± 0.006	0.011 ± 0.009	0.002 ± 0.004	0.010 ± 0.000	

Table 1: Summary data from the transplantation of expanded cells in the NOD/SCID mouse model. After the two-step expansions were complete, a sample of cells from the Stemline™ and Stemline™ II cultures were prepared for transplantation into NOD/SCID mice. These different doses of cells were chosen for injection into the mice. Due to an underestimation of the number of cells required for successful transplantation following *in vitro* expansion, a limiting dilution of cells was not reached. This is currently being repeated with lower doses of cells. However, a high percentage of the mice survived transplantation with cells expanded from both media (higher with Stemline™ II), all of which contained a small number of CD45⁺ human cells as proof of engraftment (an even smaller number of which were also CD34⁺). Both media expanded enough functional, early progenitors to achieve long-term engraftment. The higher survival rate in Stemline™ II may be explained by the higher levels of the late progenitors required for early engraftment and amelioration of the post-transplant nadir in mature myeloid cells.

Figure 2: Stemline™ and Stemline™ II bench-scale expansion of CD34⁺ cells from bone marrow compared to other HSC media. Assays were set up as in Figure 1. The cells were counted on day 14 and the fold increase was determined by (cells_{day 14}/cells_{day 0}). For the expansion of bone marrow CD34⁺ cells, Stemline™ performed as well as, or better than, the other competitors. However Stemline™ II was vastly superior to the other commercially available serum-free HSC media, giving approximately 5 fold more total nucleated cells (n = 5 donors; p<0.05). Flow cytometry on the expanded cells reveals that Stemline™ II also expands a greater number of CD34⁺ stem cells (both CD38⁺ and CD38⁻). Cells/yL is normalized to the average number of cells/yL in Stemline™ + S.E.M. (n = 3 bone marrow donors).

Conclusions

Bench-scale expansions

- Stemline™ and Stemline™ II are capable of expanding CD34⁺ cells from umbilical cord blood, adult bone marrow, and mobilized peripheral blood in bench-scale expansions. Both Stemline™ media expand CD34⁺ cells from all three sources better than the serum-free commercially available competitors.

Clinical-scale expansions

- Both Stemline™ media were able to expand CD34⁺ cells from cord blood in a clinical-scale expansion.
- Flow cytometric analysis of the clinical-scale expansions reveals that Stemline™ and Stemline™ II expand comparable numbers of early progenitor cells (CD34⁺/CD38⁻).
- Stemline™ II also has the additional benefit of a higher capacity for the expansion of the CD34⁺/CD38⁺ late progenitors required for short-term engraftment.
- The ratio of early to late progenitors shifts after the culture is expanded in Stemline™ II. In the expanded culture the percentage of the CD34⁺ cells that are also CD38⁺ is greater than in the starting material.
- CFU-E, CFU-GM, and CFU-GEMM are all present in the CFU assays of cells cultured in Stemline™ II (data not shown).
- Cells expanded in both Stemline™ and Stemline™ II were capable of repopulating NOD/SCID mice using serial passage, demonstrating self-renewal of expanded cells.

Acknowledgements

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