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Improved immunomagnetic enrichment of CD34(+) cells from umbilical cord blood using the ClinMACS cell separation system

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Abstract

Background—CD34⁺ enrichment from cord blood units (CBU) is increasingly used in clinical applications involving ex vivo expansion. The CliniMACS instrument from Miltenyi Biotec is a cGMP immunomagnetic selection system primarily designed for processing larger numbers of cells; a standard Tubing Set (TS) can process a maximum of 60 billion cells, while the larger-capacity Tubing Set (LS) will handle 120 billion cells. By comparison, most CBU contain only 1–2 billion cells, raising a question as to the optimal tubing set for CBU CD34⁺ enrichment. We compared CD34⁺ cell recovery and overall viability after CliniMACS processing of fresh CBU with either TS or LS tubing sets.

Methods—46 freshly collected CBU (< 36 hours) were processed for CD34⁺ enrichment; 22 consecutive units were selected using TS and a subsequent 24 processed with LS. Cell counts and immunophenotyping were performed pre- and post-selection to assess TNC, viability, and CD34⁺ cell content.

Results—Two sample t-tests of mean CD34⁺ recovery and viability revealed significant differences in favor of the LS tubing set (CD34⁺ recovery: LS=56%, TS=45%, p=0.003; viability: LS=74%, TS=59%, p=0.011). Stepwise linear regression, considering pre-processing unit age, viability, TNC, and CD34⁺ purity, demonstrated statistically significant correlations only with tubing set used and age of the unit.

Discussion—For CD34⁺ enrichment from fresh CBU, LS provided higher post-selection viability and more efficient recovery. In this case, lower maximum TNC specification of TS was not predictive of better performance. The same may hold for smaller-scale enrichment of other cell types with the CliniMACS instrument.

Keywords

CD34; CliniMACS; cord blood; enrichment; immunomagnetic selection

Introduction

While umbilical cord blood represents a readily available source of hematopoietic stem cells (HSC) for transplantation, the low cell dose available in a cord blood graft correlates with a significant delay in hematopoietic recovery and a higher risk of primary graft failure(1, 2). In order to provide higher absolute numbers of HSC for infusion, significant efforts are

Declaration of Interest:

The authors report no conflicts of interest in connection with this manuscript.

focused on developing clinically relevant *ex vivo* expansion methodologies, many of which require an initial enrichment of the progenitor population (3–7). A number of devices and reagents are commercially available for enrichment of CD34⁺ or CD133⁺ populations; however, these devices must comply with current Good Manufacturing Practices (cGMP) if they are to be used in the manufacturing process of cellular therapies (8).

With the discontinuation of the Isolex 300i Magnetic Cell Selection System by Baxter Healthcare, the CliniMACS Cell Separation System from Miltenyi Biotec is currently the only cGMP-grade cell separation system available for immunomagnetic separation of CD34⁺ progenitor cells from blood products. The CliniMACS system was originally designed for selection of CD34⁺ cells from peripheral blood apheresis collections. Miltenyi offers two clinically compliant tubing sets, the Tubing Set-TS (TS) and the Tubing Set-LS (LS), for use with the CliniMACS system based on the TNC of the starting material. Per manufacturer recommendations, TS sets are for processing up to 0.6×10^9 CD34⁺ cells from a maximum TNC of 60×10^9 , and LS sets are intended for larger-scale enrichments of up to 1.2×10^9 CD34⁺ cells from 120×10^9 TNC(9). Schumm *et al.* have reported no significant differences in the enrichment performance of the different tubing sets when used to enrich CD34⁺ cells from apheresis collections that were within the recommended TNC ranges(10). In contrast, cord blood units (CBU) typically contain only one to two billion cells. However no lower-limit specifications are described for such smaller-scale selections from CBU or other low TNC sources with the different tubing sets, although a logical presumption would be that the TS tubing set would be more appropriate for those products that have 10–100 fold lower TNC than a typical apheresis collection.

In support of ongoing clinical trials using *ex-vivo* expanded cord blood we have been performing CD34⁺ cell selection with the CliniMACS system, and have evaluated enrichment performance using both TS and LS sets with freshly collected CBU (no older than 36 hours old). Here we present a consecutive cohort unpaired comparison of CD34⁺ cell selection efficiencies from 46 CBU; the first 22 enriched using the TS set and the next 24 with the LS set on the CliniMACS instrument.

Methods

CBU Collection and Processing

Fresh, non-cryopreserved, RBC-replete CBU were obtained from the Puget Sound Blood Center cord blood bank (Seattle, WA) after normal delivery from full-term births (36 weeks gestational age) using standard collection procedures. All collections were performed with maternal informed consent and donor IDM testing at multiple hospitals in Washington and Hawaii, USA under Institutional Review Board (IRB) approval. All units were transported and stored at room temperature (RT).

Upon receipt, CBU were sampled for cell counts and immunophenotyping by flow cytometry prior to processing; units that contained less than 3.5×10^6 viable CD34⁺ cells were not used. The entire volume of a single CBU was then transferred to one of two plastic transfer packs connected by sterile tubing, and RBC depleted by gravity hydroxyethylstarch(HES) sedimentation for 60–90 minutes at RT. The RBC-reduced CBU was then processed for CD34⁺ selection with CliniMACS CD34 Reagent (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) following recommended Miltenyi protocols, with the following exceptions: CliniMACS buffer (Miltenyi Biotec) was supplemented with 2% human serum albumin; cell labeling was performed at RT in a 50mL tube, and cells were resuspended to a total volume of 5mL prior to the addition of IVIG and CliniMACS CD34 Reagent; CliniMACS CD34 Reagent was added at a ratio of 20uL reagent for every 10^8 CB cells; final loading volume for CD34⁺ selection was 100mL. This modified labeling

procedure was developed by our laboratory to compensate for the reduced TNC and total viable CD34⁺ cell numbers in CBU as compared to apheresis collections; a limited number of experiments were conducted employing the Miltenyi AutoMACS selection device to verify that this procedure would result in good selection performance (data not shown). As indicated by the manufacturer, TS tubing sets were run with the “CD34 Selection 1” program while LS tubing sets were run with the “CD34 selection 2” program(9). All selections were performed on a CliniMACS Plus instrument. After separation, the CD34⁺-enriched fraction was transferred to a 50mL centrifugation tube, centrifuged to remove buffer, and resuspended in a volume of 2–5mL of culture medium. Samples were then taken for cell counting and analysis by flow cytometry for CD34⁺ purity and overall viability.

Flow Cytometry

The pre-processing CBU samples were incubated with ammonium chloride red cell lysis buffer for 10 minutes at RT and then washed with PBS containing 2% human AB serum. Cells were then stained with FITC-conjugated anti-CD34, APC-conjugated anti-CD45 (BD Biosciences, San Jose, California, USA), and 7-AAD (Beckman Coulter, Marseille, France). The specific fluorochrome combinations used to evaluate CD34⁺ cell content were based on protocols developed and used by our laboratory. The stained samples were run on Becton Dickinson flow cytometers (BD Biosciences) and the data analyzed using FlowJo v9.0.1 software (Tree Star, Inc., Ashland, OR).

CD34⁺ Cell Enumeration

TNC from samples of CBU were determined using a hemocytometer, and the percent CD34⁺ and viability were determined by flow cytometry analyses and used to calculate total CD34⁺ cell numbers in the CBU before and after processing.

Statistical Methods

The unmanipulated CBU characteristics age (in hours, from time of collection to time of processing), volume, TNC, viability (by 7-AAD flow cytometry), CD34 purity (defined as pre-processing viable CD34⁺ cells divided by viable and non-viable TNC), total CD34⁺ cell number, and viable CD34⁺ cell number were considered explanatory variables that could affect selection efficiency in addition to the tubing set used. The post-selection outcome measures were TNC viability, viable CD34 purity (defined as viable CD34 divided by viable and non-viable TNC), and viable CD34⁺ cell recovery (measured as the ratio of post-selection viable CD34⁺ cells to pre-processing viable CD34⁺ cells). The statistical significance of differences between unmanipulated CBU characteristics and outcome measures were determined using t-tests.

Multiple linear regression models were used to adjust for the effects of explanatory variables on the outcome measures. Selection of variables for the regression model was accomplished by first examining all independent variables graphically using scatterplots in conjunction with correlation analysis. This allowed identification of variables with potential co-linearity effects. Stepwise regression was used to develop the final model for each outcome. Partial residual plots with locally weighted regression curves were used to demonstrate fitness of the linear model. All statistical analyses were performed using Stata/IC 11.0 (StataCorp, College Station, TX).

Results

Mean post-selection CD34⁺ cell recovery, CD34⁺ purity, and viability were significantly higher for those units processed using the LS set as compared to results from TS processed units (Figures 1 and 2, Table I). Groupwise comparisons of unmanipulated unit

characteristic means revealed that TNC was significantly different between the two groups (Table I). TNC was significantly correlated with both volume and CD34⁺ number (correlation coefficients 0.52 and 0.60, respectively). This made logical sense – more blood should mean higher TNC, and higher TNC should mean more CD34⁺ cells. To reduce co-linearity effects, we removed CD34⁺ cell number from the regression model. Adjusted regression using the variables tubing set, TNC, age, viability and CD34⁺ cell purity demonstrated statistically significant effects of the tubing set used and the age of the unit, with p values of 0.009 and 0.04, respectively (Table II). Subset analysis by age was performed using adjusted linear regression of CD34⁺ recovery by tubing set, TNC, unmanipulated viability, and CD34⁺ cell purity. For units analyzed within the median time of 20 hours (n=26), only the variable tubing set was statistically significantly associated with CD34⁺ recovery (p=0.007); however, for units > 20 hours (n=20), the association of CD34⁺ recovery with the tubing set used was not statistically significant (p=0.22). A test for interaction to determine whether the effect of the tubing set on CD34⁺ recovery was the same across these age groups resulted in p=0.11.

An additional finding of univariate analysis was that the post-selection viability was significantly higher when the LS rather than the TS was used (Table I). An adjusted linear regression model was applied to evaluate the effect of the tubing set and unmanipulated unit age, viability, TNC, and CD34⁺ purity on post-selection viability. The LS tubing set, age of the unit at processing, and the unmanipulated unit viability were associated with higher post-selection viability, although the tubing set association did not reach statistical significance (p=0.064, 0.036, and 0.046, respectively; Table III). Adjusted linear regression separately in units ≤ 20 hours and those > 20 hours revealed that the only significant association with post-selection viability was the effect of the tubing set for units ≤ 20 hours (p=0.003), but not for units > 20 hours (data not shown).

Discussion

Earlier attempts to develop clinically beneficial ex vivo expansion methods of HSC were not largely successful, however newer methods of ex vivo expansion with CBU are showing promise in early phase clinical trials(11, 12). In some cases, continued success of this approach is dependent upon an upfront efficient and reliable enrichment of progenitor cells from cord blood or other low TNC stem cell sources intended for ex vivo manipulation.

Our data demonstrate that CD34⁺ cell enrichment from fresh single CBU using the Miltenyi CliniMACS may be significantly improved using the LS tubing set instead of the TS tubing set. For our specific data set, the LS tubing set, independent of any other explanatory variables, resulted in higher post-selection viability and viable CD34⁺ recovery for units ≤ 20 hours old, but not for units older than 20 hours, although our data set contains a small number of observations when divided in this fashion. It is possible that with a larger data set we might see age-indiscriminant improvements; nevertheless, even in our data set, the LS tubing set trended toward improved enrichment compared to the TS for units > 20 hours. Our regression analysis, combined with the interaction test, demonstrates that the LS tubing set is associated with higher viable CD34⁺ recovery in all samples; however, the effect of the tubing set may vary by age because of the trend toward a greater magnitude of improvement in recovery for the age ≤ 20 hour group. Further studies may be warranted to better understand the effect of unit age on recovery.

Our data also demonstrates statistically significantly higher in CD34 purity when using the LS set as compared to the TS set. This finding is likely influenced by the higher post-selection viability in the LS group, since non-viable cells are considered a contaminant in

our calculation of CD34 purity and suggests that use of the LS tubing set results in better clearing of debris from the enriched fraction.

Our observed post-selection viability is lower compared to other reported post-selection viabilities obtained processing CBU with the CliniMACS(13). One explanation for this is that previous studies typically utilized ficoll-gradient separation prior to enrichment, or used previously cryopreserved CBU as a starting material. As opposed to ficoll, the HES sedimentation procedure that we utilize to deplete RBC from the fresh CBU may retain more non-viable cells, which end up contaminating the final enriched product. Nevertheless, the LS provided a statistically significant improvement in viability over the TS with our process, and may improve the clearing of debris from enriched products when using other preparation methods, or when other cell sources are processed with the CliniMACS device.

The primary difference between the LS and TS tubing sets is the size of the separation columns, with the LS column being wider and taller to accommodate twice the number of labeled target cells. The “CD34⁺ Selection 2” program adjusts for the larger column size by increasing the loading speed to keep the flow rate through the column at a rate similar to the “CD34 Selection 1” program for the TS. This results in a decreased run time for enrichment of smaller volumes using the LS set as compared to the TS. The larger column size also necessitates larger buffer volumes during the column wash cycles. We hypothesize that these differences in loading flow rate and washing volumes, along with the higher binding capacity, may account for better retention of the CD34⁺ cells and more efficient removal of non-CD34⁺ cells from the LS columns, resulting in higher purity, viability, and recovery. It seems likely that similar improvements might be achieved with other types of cell selections, and we have some preliminary data that CD8 enrichment is also better using the LS tubing set (data not shown).

Based on these results it is likely that the LS tubing set, when compared to the TS tubing set, may also provide improved recovery when limited numbers of TNC from other cell sources such as bone marrow or small draws of peripheral blood are processed. Furthermore, as immunotherapy becomes more prominent in the cord blood transplant setting, additional validation of the LS tubing set for enhanced enrichment of other cell types such as CD56⁺ NK cells or specific naïve/memory or Regulatory T-cell subsets from smaller starting sample volumes will need to be explored.

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Figure 1. Scatter plot of percent recovery of CD34⁺ cells after CliniMACS enrichment using the TS and LS tubing sets, respectively. Error bars represent mean $\pm$ SEM; $n=22$ for TS, $n=24$ for LS; $P=0.003$.

Figure 2. Scatter plot of percent viability of cells after CliniMACS CD34⁺ enrichment using the TS and LS tubing sets, respectively. Error bars represent mean $\pm$ SEM; $n=22$ for TS, $n=24$ for LS; $P=0.011$.

Summary of CBU characteristics analyzed prior to processing and after CD34⁺ enrichment with the CliniMACS device using the TS and LS tubing sets.

Table 1

	TS Tubing Set (<i>n</i> = 22) Mean ± SEM	LS Tubing Set (<i>n</i> = 24) Mean ± SEM	<i>P</i> -value
Unmanipulated Unit	Age (hours)	18.75 ± 1.5	0.097
	Volume (ml)	118 ± 4	0.786
	Viability (%)	96 ± 0.9	0.573
	TNC (x10 ⁸)	14.3 ± 1.1	0.029
	CD34 purity (%)	0.42 ± 0.10	0.801
	CD34 count (x10 ⁶)	7.7 ± 0.76	0.076
Post-enrichment	Viability (%)	74 ± 4.1	0.011
	CD34 recovery (%)	56 ± 2.5	0.003

Data values shown are mean ± SEM. P-values represent results of t-tests.

Results of multiple linear regression of CD34⁺ recovery after CliniMACS enrichment examining the tubing set type, age of unit in hours, pre-processing CD34⁺ purity, preprocessing CBU TNC and pre-processing viability.

Table II

CD34 Recovery	VIF	Mean Difference Between Groups	P-value	Confidence Interval
Tubing Set	1.20	0.109	0.003	0.04–0.18
Unit Age	1.14	0.004	0.036	–0.01–0.00
TNC/ml	1.07	–1.04E-08	0.064	–2.15E-06–6.34E-10
Mean VIF	1.14			

Results of multiple linear regression of cell viability after CliniMACS enrichment examining the tubing set type, age of unit in hours, pre-processing CD34⁺ purity, preprocessing CBU TNC and pre-processing viability.

Table III

Post-selection Viability	VIF	Mean Difference Between Groups	P-value	Confidence Interval
Tubing Set	1.10	0.12	0.041	0.01–0.23
Unit Age	1.07	–0.01	0.047	–0.01–0.00
Pre-selection Viability	1.04	1.05	0.028	0.12–1.99
Mean VIF	1.07			