

Optimization of Isolation and Further Molecular and Functional Characterization of SSEA-4⁺/Oct-4⁺/CD133⁺/CXCR4⁺/Lin^{neg}/CD45^{neg} Very Small Embryonic-Like (VSEL) Stem Cells Isolated from Umbilical Cord Blood

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Background

Several lines of evidence support the hypothesis that pluripotent stem cells (PSCs) reside in human tissues. Recently, we identified such primitive population of Very Small Embryonic-Like stem cells (VSELS) in umbilical cord blood (CB). These CB-VSEL stem cells: i) are very small in size (<6µm); ii) SSEA-4⁺/Oct-4⁺/CD133⁺/CXCR4⁺/Lin^{neg}/CD45^{neg}; iii) respond robustly to stromal derived factor 1 (SDF-1) gradient; and iv) possess relatively large nucleus filled out with primitive euchromatin.

There is a need to develop more optimal purification strategies of CB-VSELS for potential clinical applications.

The efficiency of recovery of CB-VSELS from fresh as well as from frozen CB units processed with different isolation protocols is unknown.

Objectives

To examine effect of i) different methods of red blood cells (RBCs) depletion from CB and ii) routine procedures employed for CB volume reduction before storage/freezing - on recovery of CB-VSELS.

To establish optimal CB processing protocol that will allow for maximal CB-VSELS survival/recovery.

To better determine morphological characteristics of different sub-populations of CB-VSELS.

Experimental Protocols

For optimizing the isolation procedure of CB-VSELS, umbilical cord blood has been collected from healthy donors. RBCs were removed by using two different protocols: 1) lysis employing hypotonic solution of ammonium chloride; 2) centrifugation in gradient of Ficoll-Paque (Fig.1A). These RBCs depletion procedures resulted in two distinct cell fractions: 1) total CB nucleated cells (TNCs) and 2) CB mononuclear cells (MNCs), respectively.

To identify CB-VSELS population in both fractions, cells were stained for presence of hematopoietic lineage markers (Lin), CD45 and CD133 and subsequently analyzed with MoFlo cell sorter (Beckman Coulter) to obtain the percent content of CB-VSELS (Fig.1B). The absolute numbers of CB-VSELS were computed based on the percent content of these cells and total number of cells in each fraction obtained by employing various isolation protocols.

The presence of CD133⁺/Lin^{neg}/CD45^{neg} CB-VSELS in both isolated fractions as well as morphological features of CB-VSELS, including expression of primitive/ pluripotent markers (CD34, SSEA-1, Oct-4), cell size and nuclear to cytoplasmic (N/C) ratio were analyzed with ImageStream system (Amnis Corp.).

To investigate the post-isolation viability of TNCs and MNCs as well as Annexin V (AnV) binding phenomenon related to RBCs-derived microvesicles (MV) transfer, both fractions were stained for CB-VSELS-related antigens (Lin, CD45, CD133) and Glycophorin A (GlyA) followed by incubation with AnV and 7-aminoactinomycin D (7-AAD). In some experiments two fractions of CB-derived total CD34⁺ cells were sorted based on the AnV binding to evaluate their functionality *in vitro* by performing hematopoietic clonogenic assay.

For analysis of the presence of CB-VSELS in frozen CB units, we evaluated the following samples obtained from Cord Blood Bank: 1) freshly collected CB; 2) concentrate of CB cells before the freezing procedure; 3) frozen concentrate of CB cells. The content of CB-VSELS was calculated by flow cytometry.

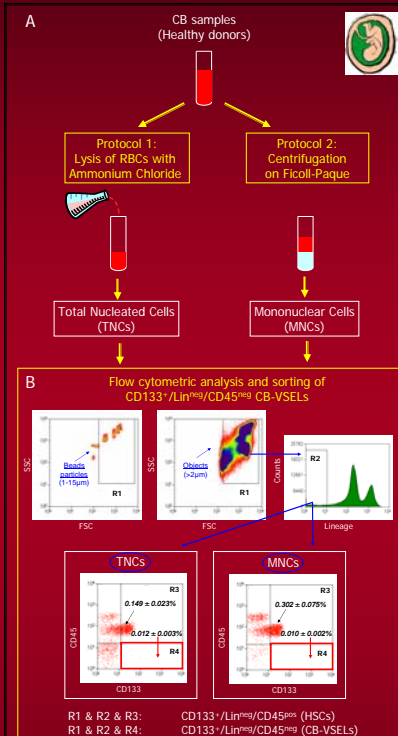


Figure 1. Panel A - Experimental procedures for depletion of RBCs to obtain TNCs or MNCs fractions. CB-VSELS were enumerated by FACS (Fluorescence Activated Cell Sorting). **Panel B** - Representative gating strategy for flow cytometric analysis and FACS sorting of CB-VSELS and hematopoietic stem cells (HSCs). Percentages show the average content of CB-VSELS and HSCs in both cellular fractions (Mean±SEM).

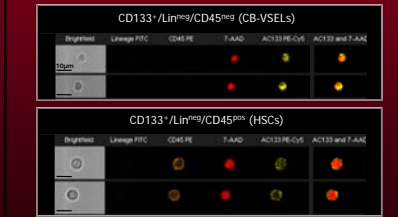


Figure 2. Images of CB-derived VSELS and HSCs obtained by ImageStream system. Each photo shows brightfield image of the cell, nuclear image after staining with 7-aminoactinomycin D (7-AAD) and images related to the expression of surface markers: Lin (green), CD45 (orange) and CD133 (AC133; yellow). The scale bars show 10µm.

Results

A. Preparation of CB-derived cells using lysis of RBCs is superior to Ficoll-Paque separation and results in higher yield of CB-VSELS

Using flow cytometry, we established that a percentage of CD133⁺/Lin^{neg}/CD45^{neg} CB-VSELS were similar in both cellular fractions isolated after centrifugation on Ficoll-Paque and after lysis of RBCs (0.010±0.002% vs. 0.012±0.003%, respectively; *P*=NS). However, when absolute numbers of CB-VSELS isolated from 1ml of CB was computed based on the percent contents and absolute number of all cells isolated by each protocol (Fig. 3A), we found that 59.2±7.2% of CB-VSELS were lost when centrifugation on Ficoll-Paque was employed. We observed decreased absolute numbers of CB-VSELS obtained from 1ml of CB after isolation on Ficoll-Paque as compared to lysis of RBCs by hypotonic ammonium chloride (505.6±91.4 vs. 1259.7±355.1, respectively; *P*<0.05) (Fig.3B).

At the same time we observed a slightly higher number of CD133⁺/Lin^{neg}/CD45^{neg} HSCs among MNCs isolated with Ficoll-Paque as compared to TNCs obtained after lysis of RBCs (0.302±0.075% vs. 0.149±0.023%, respectively; *P*=NS). Based on this, HSCs could be effectively isolated by both RBCs depletion methods as indicated by similar total number of cells obtained from 1ml of CB processed on Ficoll-Paque gradient or lysed by hypotonic ammonium chloride solution (16079.1±3997.6 vs. 15492.6±2399.1, respectively; *P*=NS) (Fig.3B).

Direct ImageStream analysis of CB-VSELS and HSCs including the size and N/C ratio of these two populations confirmed that CB-VSELS are much smaller than HSCs (6.75±1.04 vs. 8.12±1.58µm, respectively) and possess higher N/C ratio (0.55±0.14 vs. 0.40±0.18, respectively) (Fig.4).

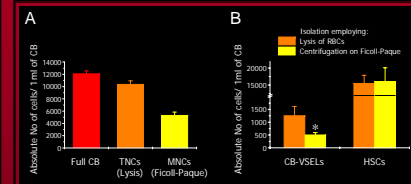
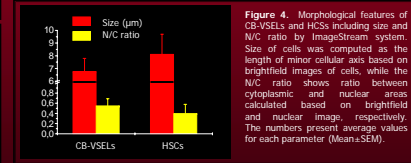


Figure 3. Panel A - Total number of cells obtained from 1ml of CB after isolation with both RBC-depletion protocols as compared to fresh CB samples. **Panel B** - Absolute numbers of CB-VSELS and HSCs that can be obtained from TNCs (after lysis of RBCs) and MNCs (after Ficoll-Paque separation) isolated from 1ml of CB. The values present Mean±SEM (P<0.05; N=5).



B. Routine CB units processing/volume depletion by currently employed protocols may lead to unwanted loss of CB-VSELS

Flow cytometric analysis revealed a significant loss (42.5±12.6%) of CD133⁺/Lin^{neg}/CD45^{neg} CB-VSELS in the concentrates of CB cells processed/prepared for storage/freezing by employing routine volume depletion strategy. At the same time only 26.67±12.5% of CD133⁺/Lin^{neg}/CD45^{neg} HSCs were lost during this preparation (Fig.5).

On the other hand, CD133⁺/Lin^{neg}/CD45^{neg} CB-VSELS are more resistant to freezing and thawing procedures as compared to CD133⁺/Lin^{neg}/CD45^{neg} HSCs. Accordingly, we observed that while about 82.66±17.31% of CB-VSELS were recovered after thawing procedure, only 65.00±12.2% of HSCs were recovered at the same time (Fig.5).

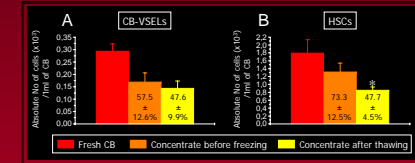


Figure 5. Panels A and B show the recovery of CB-VSELS and HSCs from CB units prepared with routine procedures for storage as well as after their thawing. The values represent the average absolute numbers of both populations (x10⁷) obtained from processing of 1ml of CB (Mean±SEM; N=5; P<0.05, when compared with fresh CB). The percentages put inside the bars are calculated as percentage of recovery of initial number of CB-VSELS and HSCs present in initial fresh CB samples.

C. CD133⁺/Lin^{neg}/CD45^{neg} CB-VSELS are enriched in primitive subpopulations expressing markers of pluripotent stem cells

In the next step, by employing ImageStream analysis, we investigated various subpopulations of CB-VSELS that express stemness markers including CD34, Oct-4 and Nanog. We employed nuclear staining, and analysis was performed only on nucleated objects with exclusion of anucleated cell debris. We investigated the percent content of three sub-fractions of CB-VSELS (Fig. 6A), their size (Fig. 6B) and N/C ratio (Fig. 6C) as well as the content of very small cells (<6µm) among each population (Fig.6D). We found that the subpopulation of CD34⁺/AnV⁺/GlyA⁺ CB-VSELS that expresses embryonic antigen SSEA-4 occurred to be the rarest, exhibits the smallest cell size and the highest N/C ratio and contains the highest number of very small cells that may indicate their most primitive pluripotent nature (Fig.6). The images of representative cells are shown in Fig. 7.

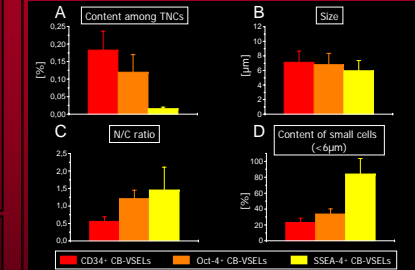


Figure 6. Panels A-D show the percent content and morphological features of primitive subpopulations of CD133⁺/Lin^{neg}/CD45^{neg} CB-VSELS characterized by co-expression of CD34, Oct-4 and SSEA-4 antigens. The values represent the average numbers calculated by ImageStream system from ten independent experiments (Mean±SEM).



Figure 7. Representative images of CB-VSELS subpopulations by ImageStream system. Each photo shows brightfield image of the cell, nuclear image after staining with 7-AAD and expression of surface and intranuclear markers. The scale bars show 10µm.

D. Microvesicles generated during lysis of RBCs transfer phosphatidylerine to the CB-VSELS

During viability analysis of CB-VSELS isolated by different protocols, we found to our surprise that high number of CB-VSELS as well as some HSCs present among TNCs (obtained after lysis of RBCs) express phosphatidylerine and bind Annexin V (47.3±9.8 and 12.9±5.9%, respectively) (Fig. 8A). This could be a sign that these cells undergo apoptosis. However, when we lysed RBCs before the staining the number of Annexin V⁺ cells was reduced to 14.1±7.6 and 3.1±1.0% for CB-VSELS and HSCs, respectively (Fig. 8A). We asked if this unexpected effect could be explained by a transfer of phosphatidylerine from lysed RBCs to CB cells. To address this issue better, we stained CB cells for expression of GlyA and found significant decrease of Annexin V⁺/GlyA⁺ CB-VSELS and HSCs when the staining was performed before the lysis of RBCs (Fig. 8B). Finally to exclude involvement of apoptosis, we sorted from CB-TNCS a population of CD34⁺/AnV⁺/GlyA⁺ TNCs and found that these cells give rise to comparable number of colonies as CD34⁺ cells sorted from non-lysed fraction of MNCs (Fig. 8C).

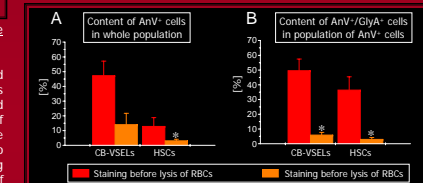


Figure 8. Panel A - Content of AnV⁺ cells among CB-VSELS and HSCs before and after lysis of RBCs. Panel B - Content of AnV⁺/GlyA⁺ cells among CB-VSELS and HSCs before and after lysis of RBCs. Panel C - Clonogenic potential of purified fraction of CD34⁺/AnV⁺/GlyA⁺ sorted after lysis of RBCs as compared to CD34⁺ cells isolated by Ficoll-Paque. The numbers represents average values (Mean±SEM).

Summary

CB processing procedures based on depletion of red blood cells (RBCs) by centrifugation on Ficoll-Paque gradient or volume reduction prior to storage/freezing led to significant loss of CB-VSELS.

Lysis of RBCs occurred to be most optimal RBCs depletion method that allows for the highest recovery of CB-VSELS.

Fraction of CB-VSELS isolated after lysis of RBCs is enriched for most primitive, pluripotent CB cells expressing CD34, Oct-4 and SSEA-4 antigens.

During RBCs lysis, some molecules present in RBCs' membrane (including phosphatidylerine and GlyA) may be transferred to CB-VSELS by microvesicles and are responsible for false positive staining.

Conclusions

The procedures of CB processing should be carefully revised and optimized in order to preserve some very small and dense primitive stem cells that are present in processed CB units - for example CB-VSELS.

The microvesicles generated during lysis of RBCs may transfer phosphatidylerine and GlyA to CB-VSELS. Transfer of these antigens have to be considered for example during Annexin V viability staining.