Optimised cord blood sample selection for small-scale CD34+ cell immunomagnetic isolation

ANA-MARÍA PERDOMO-ARCINIEGAS and JEAN-PAUL VERNOT

Cellular and Molecular Physiology Group, Physiology Division, Faculty of Medicine, Universidad Nacional de Colombia, Bogotá, Colombia

Received September 14, 2011; Accepted November 24, 2011

DOI: 10.3892/mmr.2011.733

Abstract. Haematopoietic stem cells (HSCs) are defined as multipotential cells, capable of self-renewal and reconstituting *in vivo* the haematopoietic compartment. The CD34 antigen is considered an important HSCs marker in humans. Immunomagnetic isolation, by targeting CD34 antigen, is widely used for human HSC separation. This method allows the enrichment of human HSCs that are present at low frequencies in umbilical cord blood (CB). Immunomagnetic CD34+-cell isolation reproducibility, regarding cell yield and purity, is affected by the CD34+ cell frequency and total cell numbers present in a given sample; CB HSC purification may thus yield variable results, which also depend on the volume and density fractionation-derived cell loss of a CB sample. The uncertainty of such an outcome and associated technical costs call for a cost-effective sample screening strategy. A correlation analysis using clinical and laboratory data from 59 CB samples was performed to establish predictive variables for CD34+-immunomagnetic HSCs isolation. This study described the positive association of CD34+-cell isolation with white and red cell numbers present after cell fractionation. Furthermore, purity has been correlated with lymphocyte percentages. Predictive variable cut-off values, which are particularly useful in situations involving low CB volumes being collected (such as prevalent late umbilical cord clamping clinical practice), were proposed for HSC isolation sampling. Using the simple and cost-effective CB sample screening criteria described here would lead to avoiding costly inefficient sample purification, thereby ensuring that pure CD34+ cells are obtained in the desired numbers following CD34 immunomagnetic isolation.

Correspondence to: Dr Jean-Paul Vernot, Cellular and Molecular Physiology Group, Physiology Division, Faculty of Medicine, Universidad Nacional de Colombia, Bogotá, DC, Colombia
E-mail: jpvernoth@unal.edu.co

Key words: haematopoietic stem cells, cord blood selection, immunomagnetic isolation, CD34

Introduction

Haematopoietic stem cells (HSCs) are the best characterised tissue-specific stem cells with more than four decades of basic research and clinical applications (1). These cells are present in different frequencies in umbilical cord blood (CB), mobilised peripheral blood and bone marrow (0.1-0.6, 0.3-4.5 and 0.8-4% nucleated cells, respectively) (2,3). Mononuclear cell separation by low-density cell fractioning, CD34+ immunomagnetic isolation (IMI) has been regularly used for HSCs purification (4,5). CD34+ cells may be separated from frozen and freshly isolated small-scale samples, mainly for basic research purposes (6-9). Studies using G-CSF mobilised peripheral blood showed CD34+ cell IMI reproducibility only when the CD34+-cell frequency of a sample exceeded 0.65% (4). Peripheral blood CD34+-cell enrichment by IMI varies from 38.6 to 87.1% (54.4±12.3%), as CD34+ cells are present at low frequencies (0.18±0.052%) among leukocytes (9). Uncertainty regarding CD34+ isolated cell numbers of a CB sample by IMI is therefore a consequence of CB low CD34+-cell frequencies, variable CB volumes and nucleated cell loss after cellular fractioning. CB volume collection and therefore CD34+-cell number variability depend highly on the timing of umbilical cord clamping (10). Given that CD34+ labeling and IMI are costly, a cost-effective strategy is to select CB samples before purification.

A correlation analysis between CD34+-cell purification outcome and different CB donor and sample variables was performed to assess CD34+-immunomagnetic HSCs purification prediction factors. Gestational age, neonatal weight and collected CB volume, as well as low-density cell fraction (LDCF) white blood cells (WBC), red blood cells (RBC) and lymphocyte percentages, were included as independent variables. The outcome purification variables analysed consisted of total purified cells, CD34+-cell purity and corrected CD34+-cell numbers. Our results showed that the CB volumes and LDCF-WBC numbers of a sample predicted total and pure CD34+-cell numbers. Notably, it was found that higher RBC numbers and lymphocyte percentages in LDCF had better outcomes in total isolated CD34+-cell numbers and CD34+-cell purity, respectively. Furthermore, specific criteria for the main predictive variables were established to obtain a minimum of $5x10^5$ CD34+ pure cells. These results may facilitate the selection of CB samples for small-scale HSCs isolation when having limited CB volumes.

Table I. Descriptive statistics for observed variables.

	No.	Mean	SD	Min	Max
Pre-laboratory variables					
Gestational age (weeks)	56	38	12	36	41
Newborn weight (g)	57	3,042	315	2,415	3,850
CB volume (ml)	54	31	10	14	67
Laboratory variables (LDCF)					
WBC ($1x10^3$)	59	170	94	27	449
RBC ($1x10^6$)	59	490	759	47	5,600
Lymphocytes (%)	59	57	11	17	86
Outcome variables					
Total isolated cell no. ($1x10^3$)	59	776	491	62	2,275
CD34 purity (%)	59	77	18	20	97
Corrected CD34 no. ($1x10^3$)	59	629	450	22	2,116

SD, standard deviation; CB, cord blood; LDCF, low-density cell fraction; WBC, white blood cells; RBC, red blood cells.

Materials and methods

Umbilical CB samples. Human umbilical CB was obtained after full-term pregnant women attending the Instituto Materno Infantil had signed a written informed consent according to the Universidad Nacional de Comlombia's Ethical Committee's guidelines. Neonatal data were available for the processed CB samples. Late cord clamping (after 2 min) was the procedure used in almost all deliveries (93%). Earlier cord clamping was performed when an obstetrician or pediatrician so indicated. All the 59 CB samples analysed were collected *in-utero* after vaginal delivery in vacutainer tubes containing dried sodium heparin (BD, Franklin Lakes, NJ, USA). Sample volumes were measured and maintained at room temperature until processing.

CB low-density cell fraction (LDCF) isolation and automated counting. Not longer than 12 h after CB sample collection, tubes were spun at 800 g for 30 min for buffy-coat collection. The LDCF was collected from the interface after layering buffy-coat suspension onto a Ficoll-Hypaque cushion (Histopaque-1077; Sigma-Aldrich, St. Louis, MO, USA) and centrifuging at room temperature for 30 min at 500 g. The LDCF was washed three times with RPMI-1640 (Gibco). LDCF counting was performed by automatic cell counter Sysmex KX-21 (Sysmex Corporation, Kobe, Japan). Total numbers of WBC and RBC were calculated based on the final suspension volume (10 ml).

CD34+ cell purification. CD34+ cells were purified by positive selection using a MACS immunomagnetic separation system (Miltenyi Biotec, Bergish Gladbach, Germany) according to the improved manufacturer's protocol (5). Total viable recovered cell numbers were determined by Trypan blue. Fluorescence-activated cell sorting of anti-CD34APC antibody (Clone AC136; Caltag Laboratories, Invitrogen Corporation, Camarillo, CA, USA) labeled cells was used for determining cell purity. Acquisition and analysis were performed in a FACSAria II (BD). Corrected CD34+-cell number was calculated by multiplying the total viable cell number and cell purity divided by 100.

Data processing and statistical analysis. Basic descriptive statistics were calculated for all variables. Multivariate linear regression was used to test the association between independent variables (pre-laboratory and laboratory variables) and dependent variables (total isolated cell number, purity and corrected CD34+ number). $p<0.05$ was considered to indicate statistical significance. Multi-collinearity was evaluated by independently calculating the variance inflating factor for pre-laboratory and laboratory variables, a value of <2 having been obtained. The receiver operating characteristics (ROC) curves of the predictive variables were constructed to identify the best cut-off criteria. Stata MP/10.0 software (Stata Corp., College Station, TX, USA) was used to perform all analyses.

Results

Pre-laboratory, laboratory and outcome variables. Regarding new-born gender distribution, 33 infants (56%) were female and 26 infants (44%) were male. Data concerning gestational age, new-born weight and sample volume collected are summarized in Table I as pre-laboratory variables; LDCF automated counting of WBC, RBC and lymphocyte percentages are summarized in Table I as laboratory variables. Total numbers obtained after CD34 isolation, flow cytometry-assessed CD34 purity percentage and purity-corrected CD34 numbers are summarized in Table I as outcome variables.

Correlation between CB volume values and total numbers. Multivariate regression analysis was used for assessing which variables could predict isolated CD34+-cell numbers. Multivariate analysis of pre-laboratory variables revealed that only CB volume values were directly correlated with total numbers obtained after CD34 isolation ($p=0.016$), as well as with corrected CD34+-cell numbers ($p=0.05$) (Table II). Multivariate analysis of laboratory variables revealed that

Table II. Multivariate linear regression analysis.

	Total isolated cells no.	CD34 purity	Corrected CD34 no.
Pre-laboratory variables			
Gestational age (weeks)	p=0.33	p=0.13	p=0.48
	n=51	n=52	n=51
Newborn weight (g)	p=0.38	p=0.28	p=0.47
	n=51	n=52	n=51
CB volume (ml)	**p=0.016**	p=0.84	**p=0.05**
	n=51	n=52	n=51
Laboratory variables (LDCF)			
WBC ($1x10^3$)	**p=0.000**	p=0.13	**p=0.000**
	n=59	n=59	n=59
RBC ($1x10^6$)	**p=0.018**	p=0.39	**p=0.046**
	n=59	n=59	n=59
Lymphocyte (%)	p=0.37	**p=0.005**	p=0.34
	n=59	n=59	n=59

P-value and sample no. are shown. Significant p-values are shown in bold. CB, cord blood; LDCF, low-density cell fraction; WBC, white blood cells; RBC, red blood cells.

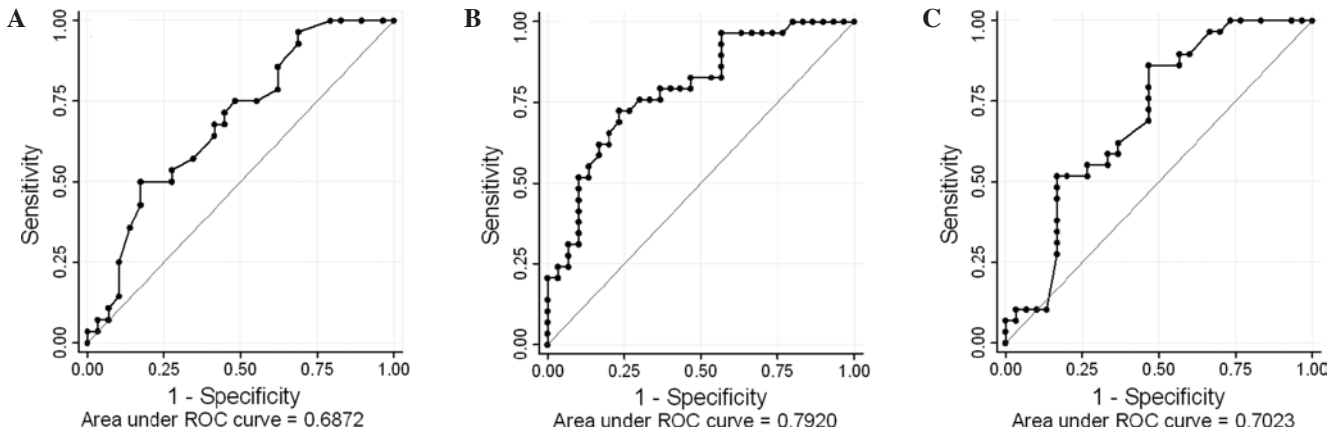

Figure 1. ROC curves of predictive variables. (A) Collected cord blood volume. (B) White blood cell numbers in low-density cell fraction (LDCF). (C) Red blood cell numbers in LDCF.

LDCF-WBC as well as LDCF-RBC were also capable of predicting total isolated cell numbers as well as corrected $CD34^+$-cell numbers (Table II). Notably, only lymphocyte percentages were directly correlated with $CD34^+$ percentages obtained after CD34 IMI isolation (Table II).

ROC curves of predictive values. After establishing a minimum of $5x10^5$, $CD34^+$ cells required for subsequent research experiments, ROC curves were constructed for predictive variables to set up appropriate cut-off points for sample selection. The CB volume ROC curve had a 0.6872 area under the curve (Fig. 1A) (0.07 standard error and 95% confidence interval of 0.548-0.826). The chosen cut-off point was 27 ml, with 75% sensitivity and 55.7% specificity (1.55 positive likelihood and 0.48 negative likelihood ratio). The ROC curve for WBC number shown (Fig. 1B) had an area under the curve of 0.79 (0.05 standard error and 95% confidence interval of 0.677-0.907). The chosen cut-off point was $194x10^6$ WBC, with 51% sensitivity and 90% specificity (5.17 positive likelihood and 0.53 negative likelihood ratio). The RBC ROC curve is shown in Fig. 1C. The chosen cut-off point for RBC number was $375x10^6$ RBC, with a 55% sensitivity and 73% specificity (2 positive and 0.6 negative likelihood ratio).

Discussion

$CD34^+$ IMI is the best method for CD34 purification, but is not infallible regarding purity and $CD34^+$-cell yield (11). This is particularly true for samples with a low $CD34^+$-cell frequency (4,9-11). Purification or enrichment of HSC at certain desired levels is a crucial initial step for basic research on HSCs (12). Since sample $CD34^+$ enumeration by antibody labeling (13) and subsequent IMI are costly and time-consuming, a cost-effective strategy would consist of selecting the samples taking into account simple clinical or laboratory variables depending on the numbers of $CD34^+$ cells required. Results of this study

have shown that purified CD34+-cell numbers were correlated with collected CB volumes, LDCF-WBC and LDCF-RBC numbers. Additionally, threshold criteria for predictive variables were established to obtain a sufficient amount of CD34+ cells needed in our study for basic research. Furthermore, our results suggested that low CD34+ purity may be prevented by avoiding processing LDCF samples from having low lymphocyte percentages.

Late-cord clamping is correlated with low CB collected volumes (14,15). This is common practice in our clinical setting due to secondary short- and long-term clinical benefits of placental transfusion for neonates (15,16). Our results are therefore applicable for low collected CB volumes. CB banking volume recommendations vary between 40 and 60 ml CB (17). If such a recommendation were to be applied, more than 80% of our samples would have to be discarded. Thus, CB samples should initially be selected using a relatively low CB volume (27 ml), thereby yielding a high sensitivity (75%) and further selection criteria after cell fractioning using LDCF-WBC numbers ($194x10^6$ WBC), raising specificity to 90% to obtain more than $5x10^5$ CD34+ cells. Complementary data of LDCF-RBC numbers may also be helpful, given that RBC in LDCF was a positive predictor factor for CD34+-cell isolation. This result was consistent with previous reports showing reduced UCB sample colony-forming ability after RBC depletion (2). LDCF-lymphocyte percentage criteria permitted discarding of samples, with a higher adhesive granulomonocytic cell portion which may decrease CD34+ purity percentages.

Other studies have been published correlating maternal, neonatal and laboratory parameters with CB-derived unpurified CD34+ enumerated cells (19,20). These studies have only enumerated CD34+ cells by flow cytometry using non-fractionated, thawed, high-volume CB samples which had previously been frozen for CB banking and clinical use. The present study provides useful simple correlation parameters and cut-off values for selecting CB samples for CD34+ IMI that are likely to improve the purification success rates, particularly when collecting samples in clinical environments where late cord clamping prevail and low CB volumes are being collected.

Acknowledgements

The authors would like to thank Mr. Jason Garry for making grammatical corrections to this manuscript, as well as Esperanza Rios at the Instituto Materno Infantil for her assistance in collecting samples. This study was financed by Colciencias (RC401-2006) and the Universidad Nacional de Colombia's DIB.

References

1. Brider D, Rossi DJ and Weissman IL: Hematopoietic stem cells: The paradigmatic tissue-specific stem cell. Am J Pathol 169: 338-346, 2006.
2. Broxmeyer HE, Douglas GW, Hangoc G, Cooper S, Bard J, English D, Arny M, Thomas L and Boyse EA: Human umbilical cord blood as a potential source of transplantable hematopoietic stem/progenitor cells. Proc Nat Acad Sci USA 86: 3828-3832, 1989.
3. Theilgaard-Mönch K, Raaschou-Jensen K, Palm H, Schjødt K, Heilmann C, Vindeløv L, Jacobsen N and Dickmeiss E: Flow cytometric assessment of lymphocyte subsets, lymphoid progenitors, and hematopoietic stem cells in allogeneic stem cell grafts. Bone Marrow Transplant 28: 1073-1082, 2001.
4. Cancelas JA, Querol S, Martin-Henao G, Canals C, Azqueta C, Petriz J, Ingles-Esteve J, Amill B and Garcia J: Isolation of hematopoietic progenitors. An approach to two different immunomagnetic methods at the lab scale. Pure Appl Chem 68: 1897-1901, 1996.
5. Kekarainen T, Mannelin S, Laine J and Jaatinen T: Optimization of immunomagnetic separation for cord blood-derived hematopoietic stem cells. BioMed Central Cell Biol 7: a30, 2006.
6. Querol S, Capmany G, Azqueta C, Gabarró M, Fornas O, Martín-Henao GA and García J: Direct immunomagnetic method for CD34+ cell selection from cryopreserved cord blood grafts for ex vivo expansion protocols. Transfusion 40: 625-631, 2000.
7. Chan SL, Choi M, Wnendt S, Kraus M, Teng E, Leong HF and Merchav S: Enhanced in vivo homing of uncultured and selectively amplified cord blood CD34+ cells by cotransplantation with cord blood-derived unrestricted somatic stem cells. Stem Cells 25: 529-536, 2007.
8. Pan Y, Nastav JB, Zhang H, Bretton RH, Panneton WM and Bicknese AR: Engraftment of freshly isolated or cultured human umbilical cord blood cells and the effect of cyclosporin A on the outcome. Exp Neurol 192: 365-372, 2005.
9. Kato K and Radbruch A: Isolation and characterization of CD34+ hematopoietic stem cells from human peripheral blood by high-gradient magnetic cell sorting. Cytometry 14: 384-392, 1993.
10. Pafumi C, Milone G, Maggi I, Russo A, Farina M, Pernicone G, Bandiera S, Giardina P, Mangiafico L, Mancari R, Calogero AE and Cianci A: Early clamping of umbilical cord blood and foetal CD34 enrichment. Acta Med Austriaca 28: 141-144, 2001.
11. De Wynter EA, Ryder D, Lanza F, Nadali G, Johnsen H, Denning-Kendall P, Thing-Mortensen B, Silvestri F and Testa NG: Multicentre European study comparing selection techniques for the isolation of CD34+ cells. Bone Marrow Transplant 23: 1191-1196, 1999.
12. Andrade PM, da Silva CA, dos Santos FF, Almeida-Porada G and Cabral JM: Initial CD34(+) cell-enrichment of cord blood determines hematopoietic stem/progenitor cell yield upon ex vivo expansion. J Cell Biochem 112: 1822-1831, 2011.
13. Gajkowska A, Oldak T, Jastrzewska M, Machaj EK, Walewski J, Kraszewska E and Pojda Z: Flow cytometric enumeration of CD34+ hematopoietic stem and progenitor cells in leukapheresis product and bone marrow for clinical transplantation: a comparison of three methods. Folia Histochem Cytobiol 44: 53-60, 2006.
14. Donaldson C, Armitage WJ, Laundy V, Barron C, Buchanan R, Webster J, Bradley B and Hows J: Impact of obstetric factors on cord blood donation for transplantation. Br J Haematol 106: 128-132, 1999.
15. Farrar D, Airey R, Law GR, Tuffnell D, Cattle B and Duley L: Measuring placental transfusion for term births: weighing babies with cord intact. BJOG 118: 70-75, 2011.
16. Hutton EK and Hassan ES: Late vs early clamping of the umbilical cord in full-term neonates: systematic review and meta-analysis of controlled trials. JAMA 297: 1241-1252, 2007.
17. Ballen KK, Wilson M, Wuu J, Ceredona AM, Hsieh C, Stewart FM, Popovsky MA and Quesenberry PJ: Bigger is better: maternal and neonatal predictors of haematopoietic potential of umbilical cord blood units. Bone Marrow Transplant 27: 7-14, 2001.
18. Solves P, Perales A, Mirabet V, Blasco I, Blanquer A, Planelles D, Larrea L, Monleon J, Carbonell-Uberos F and Soler MA: Optimizing donor selection in a cord blood bank. Eur J Haematol 72: 107-112, 2004
19. Canabarro R, Sporleder H, Gomes T, Zanatta G, Scribel L, Freitas F, Neumann J and Pranke P: Immunophenotyping evaluation, and physiological and laboratory correlations of hematopoietic stem cells from umbilical cord blood. Biocell 31: 397-403, 2007.
20. Urciuoli P, Passeri S, Ceccarelli F, Luchetti B, Paolicchi A, Lapi S, Nocchi F, Lamanna R, Iorio M, Vanacore R, Mazzoni A and Scatena F: Pre-birth selection of umbilical cord blood donors. Blood Transfusion 8: 36-43, 2010.