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Original

Aldehyde Dehydrogenase Activity as a Marker of Quality in Cryopreserved Cord Blood

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Abstract: We investigated the effects of cryopreservation on CD34＋ cells and aldehyde dehydrogenase (ALDH)-positive cells (ALDH (＋) cells) in the umbilical cord blood (UCB) of unrelated donors. Ten units of UCB were collected at the Kanagawa Cord Blood Bank from September 2009 to November 2010. These UCB units were frozen for 2 weeks or 1 year, and then assayed for quality by flow cytometry analysis and colony-forming assay. We found that both ALDH (＋) cells and the numbers of total colony-forming units and colony-forming units of granulocyte / macrophage from the isolated CD34＋ cells were significantly decreased after all cryopreservation. The numbers of ALDH (＋) / CD34＋ cells, ALDH (＋) / CD34＋CD38− cells, and total colony-forming units from the cryopreserved UCB units continued to decrease over time with cryopreservation. In contrast, levels of traditional primitive surface markers were not significantly decreased in CD34＋ cells and CD34＋ CD38− cells after cryopreservation, although this result depended on the duration of cryopreservation. These findings suggested that ALDH (＋) cells could be a new marker for assessing cryopreserved UCB quality prior to hematopoietic cell transplantation.

Key words: primitive hematopoietic stem cells, aldehyde dehydrogenase activity, cryopreservation, umbilical cord blood

Introduction

Umbilical cord blood (UCB) is used as an alternative source of progenitor cells for hematopoietic stem cell (HSC) transplantation in children and adults[1, 2]. Most of the worldwide cord blood (CB) banks cryopreserve UCB units individually in liquid nitrogen for several years and then do a rapid thaw immediately prior to hematopoietic cell transplantation (HCT). It is therefore necessary to monitor the suitability of CB stem and progenitor cell components before cryopreservation and after thawing[3, 4]. Cryopreservation is associated with a variable loss of cell viability and a subsequent decrease in the absolute numbers of nucleated cells available for infusion[5]. However, HSCs seem more resistant to cryopreservation injury among the total population of nucleated cells, and CD34＋ cells in particular have demonstrated higher viability than the overall mononucleated cell population[6]. However, while there are no significant decreases in the CD34＋ CD38− and CD34＋ cell populations after cryopreservation-thawing[7, 8],

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it is not fully understood whether a primitive subpopulation of HSCs could be maintained after cryopreservation-thawing.

CD34 expression is a significant marker of HSCs used for HCT; however, cell surface phenotypes can be altered by cell cycle progression and ex vivo manipulation[9–11]. One promising alternative strategy for identifying HSCs and progenitors is the detection of intracellular enzyme expression. Aldehyde dehydrogenase (ALDH) is one such enzyme that is expressed in HSCs, but not in mature cells. The potential benefit of evaluating ALDH expression for HCT is that the expression of this enzyme could delineate a more immature stem cell population that is preferable for long-term repopulation[12, 13]. Using fluorescence-activated cell sorting (FACS), specific cell fractions from human CB, characterized to contain ALDH by low orthogonal light scattering and bright fluorescence intensity (ALDH+), were found to be highly HSC enriched[14]. In this study, we therefore sought to determine whether ALDH could be another robust marker for HCT by examining previously uncharacterized ALDH (+) cells in UCB units cryopreserved for transplantation. We also investigated the effects of freezing duration on the UCB cellular markers.

Materials and methods

This study was performed at the Showa University Fujigaoka Hospital from September 2009 to November 2011. All protocols used for subject recruitment and UCB collection were reviewed and approved by the Ethics Committee of the Kanagawa Cord Blood Bank in Japan, and the Ethics Committee of the Showa University Fujigaoka Hospital approved the study protocol. All volunteers provided written informed consent prior to admission into the study.

A total of 10 UCB units that could not be used for HCT based on volume were obtained from the Kanagawa Cord Blood Bank for use in this study. For each sample, we counted total nucleated cells (TNCs), ALDH (+) cells, CD34+ cells, and colony-forming units (CFU), as well as assessing cell viability and determining the proportions of CD34+ cells expressing CD38 before cryopreservation and after thawing. In addition, the relationship between CD34+ CD38− ALDH (+) cells and the colony-forming units-granulocyte / macrophage (CFU-GM) in the UCB units were analyzed after thawing.

Collection of UCB units

UCB samples were obtained from normal full-term deliveries in the Department of Obstetrics and Gynecology of the Showa University Fujigaoka Hospital. Every UCB unit was collected after delivery of the neonate and ligation of the cord, and prior to the expulsion of the placenta. The cord was disinfected, the umbilical vein was punctured, and the UCB was collected into a 200-ml collection bag (CBS-20A ; Nipro Co., Osaka, Japan) containing 28 ml of citrate-phosphate-dextrose as an anticoagulant.

Processing of UCB units

Hydroxyethyl starch separation was performed according to the methods of Rubinstein *et al*[16].

Briefly, aliquots were removed for routine testing, and then 6% (wt / vol) hydroxyethyl starch (HES40 ; Nipro Co.) was added to the anticoagulated UCB to achieve a final concentration of 1.5% (1 : 3 volume ratio) and increase the red blood cell sedimentation rate. The collection bag was centrifuged (60 × g for 5 min at 10℃) to obtain a leukocyte-rich supernatant, which was then transferred into a separation bag (CBP-20D ; Nipro Co.) and recentrifuged (400 × g for 10 min at 10℃) to pellet the UCB cells. Surplus supernatant plasma was transferred into a second bag via the connecting tube. Finally, the sedimented leukocytes were resuspended in supernatant plasma to a total volume of 23 ml for use as a leukocyte concentrate. One milliliter of the leukocyte concentrate was used as the pre-freeze sample.

Cryopreservation of UCB units

The required volume of sterile, chilled 50% dimethyl sulfoxide (DMSO) in 5% (w / v) dextran 40 (Terumo, Tokyo, Japan) was slowly added to the UCB product to obtain two different final concentrations, 10% and 1%, which were then mixed and transferred to a 25-ml freezing bag (F-025 ; Nipro Co.) and maintained at 4℃ in preparation for cryopreservation. Each final UCB product was divided into two before freezing to provide a 2-week and 1-year cryopreserved sample, before placing in a controlled-rate freezer. After freezing, the bags were transferred to a liquid nitrogen storage container.

Thawing and washing of the UCB units

The UCB units were thawed and washed according to methods originally described by Rubinstein *et al*[16]. Briefly, the units were removed from liquid nitrogen storage, placed in a plastic bag, and immersed in a 37℃ water bath to thaw, before centrifugation at 400 × g for 15 min at 4℃. Approximately three-fourths of the supernatant was removed and transferred to a second bag for further centrifugation at 800 × g for 15 min at 4℃. This wash step was repeated and then the cell pellets from the two centrifugation steps were combined into 1 bag per original unit. The combined cells pellets were resuspended in phosphate-buffered saline (PBS) and finally passed through a standard blood filter (170−260 / mm) to give the final sample for evaluation.

Determination of ALDH (+) cells, CD34+ cells, and CD34+ CD38− cells using flow cytometry

We determined the percentages of ALDH (+) cells, ALDH activity, and CD34 and CD38 antigen expression levels in matched pre-freeze, 2-week, and 1-year cryopreserved samples. For all antibody staining experiments, the cell mixtures were diluted to 1 : 20 in an ammonium chloride (NH_4Cl) lysing solution, then incubated at room temperature for 5 min, and finally washed with staining media prior to FACS analysis. UCB samples were immunostained with the cells at a concentration of 10^6 cells / ml for 15 min prior to staining with bodipy-aminoacetaldehyde (BAAA). Cells with low side scatter and high BAAA staining levels were defined as Side Scatter Low ALDH Bright (ALDH (+)) cells. ALDH activity was assessed with the bodipy-based ALDEFLUOR assay (Becton Dickinson Co., Franklin Lakes,

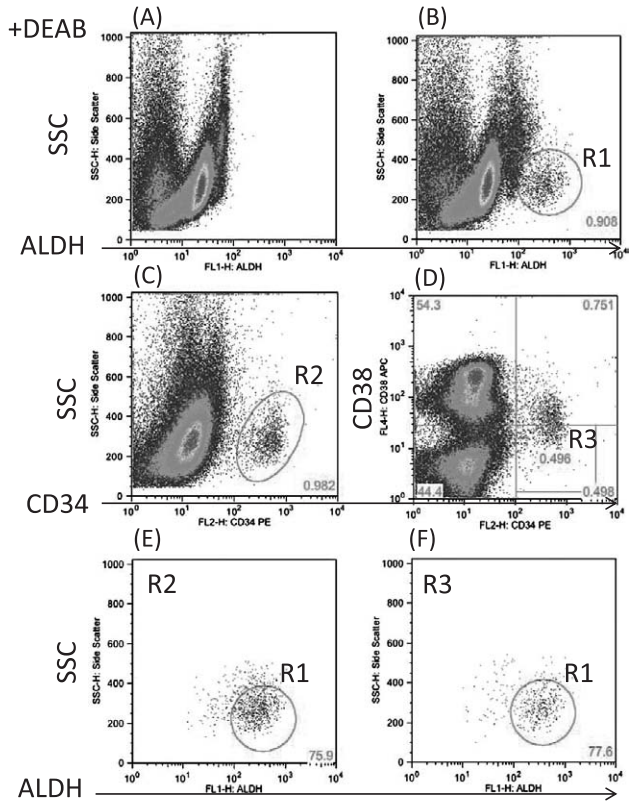

Fig. 1. Gating strategy for determining the percentages of ALDH (+), CD34+ / ALDH (+) cells, and CD34+CD38− / ALDH (+) cells. Pre-freeze and cryopreserved samples were set aside for subsequent reaction with ALDEFLUOR. Control cells were reacted with ALDEFLUOR in the presence of the ALDH inhibitor DEAB (A). The cells were reacted with ALDEFLUOR alone to establish R1 as ALDH (+) cells (B). CD34+ cells and CD34+ CD38− cells were sorted into the R2 area (C, CD34+) and R3 areas (D, CD34+ CD38−), respectively. The cells gated as R2 also expressed CD34 (E, CD34+ / ALDH (+)), while the R3 cells also expressed CD34, but not CD38 (F, CD34+ CD38− / ALDH (+)).

NJ, USA), according to the manufacturer's instructions. Diethylaminobenzaldehyde (DEAB), a potent inhibitor of ALDH activity, was used to provide a negative control. After a 30-min incubation at 37°C, the cells were centrifuged in cold PBS and suspended at 10^7 cells / ml in cold PBS for costaining with fluorescence-conjugated monoclonal antibodies, including phycoerythrin (PE)-conjugated anti-CD34 and allophycocyanin (APC)-conjugated anti-CD38. Dead cells were excluded after staining with 7-amino-actinomycin D (7AAD; 1 μg / 10^6 cells; Beckman Coulter Ins., Brea, CA, USA). Antibody- and BAAA-stained cells were analyzed on a FACSCalibur flow cytometer (Becton Dickinson) equipped with a 488-nm argon laser and a 635-nm red diode laser. Bodipy fluorescence was excited at 488 nm, and fluorescence emission was detected using a standard fluorescein isothiocyanate (FITC) 530 / 30-nm bandpass filter. FlowJo software (Becton Dickinson) was used for cytometric analysis. The gating strategy for determining the percentages of ALDH (+), ALDH (+) CD34+, and ALDH (+) CD34+ CD38− cells is shown in Fig. 1. The control cells, which were reacted with ALDEFLUOR in the presence of the ALDH inhibitor DEAB, are shown in Fig. 1A. The cells that reacted with ALDEFLUOR alone and with CD34 are in the R1 and R2 areas, respectively (Fig. 1B and 1C), while R3 represents CD34+ CD38− cells (Fig. 1D). CD34+ cells, which exhibit high ALDH activity, are shown in Fig. 1E, and Fig. 1F shows the CD34+ CD38− cells with high ALDH activity.

Colony-forming assay derived from CD34+ cells

1) Isolation of CD34+ cells from UCB

CD34+ cells were isolated on magnetic-activated cell-sorting columns with microbead-conjugated antibodies (MiniMACS system, Miltenyi Biotec, Bergisch Gladbach, Germany). The purity of the CD34+ cells after MACS sorting was determined by flow cytometry to be generally higher than 95%.

2) CFU and CFU-GM derived from CD34+ cells

To determine the number of total CFU and CFU-GM, 2500 sorted CD34+ cells were plated on 35-mm tissue culture plates containing 1.5 ml of methylcellulose medium (MethoCult GFH4434V, Stem Cell Technologies, Inc., Vancouver, BC, Canada). CFU-GM, total CFU, erythrocytes, macrophages and megakaryocytes and erythroid burst-forming units from isolated CD34+ cells were counted under an inverted microscope (Olympus, Tokyo, Japan) after 14 days. We measured the total CFU and CFU-GM colonies in a semi-solid culture assay among CD34+ cells isolated from matched pre-freeze, 2-week, and 1-year cryopreserved samples. CFU-GMs, defined as aggregates of more than 40 cells, were counted under an inverted microscope.

Statistical analysis

Data were expressed as mean ± standard deviations (SD). The statistical significance ($P<0.05$) between pre- and post-thaw samples was determined by using Student's t test for paired samples and the Mann Whitney and Wilcoxon non parametric tests for independent values when the data did not reflect a Gaussian distribution. Biostatistical analyses were performed with StatView software (version5.0; SAS institute, Cary, NC, USA).

Results

Viability of TNCs and CD34+ cells according to cryopreservation time

The success of UCB transplantation depends on the numbers of TNCs and CD34+ cells per recipient weight, and cryopreservation reportedly does not affect the viability of these cells[18]. Herein, we confirmed that neither the TNC and CD34+ cell counts nor viability was significantly decreased in the 2-week cryopreserved UCB samples compared with those in the pre-freeze samples. However, in the 1-year cryopreserved samples, while the cell counts remained comparable to those in the pre-freeze samples, the viability of both TNCs and CD34+ cells was significantly decreased compared with that in the pre-freeze samples (Table 1).

Viability of ALDH (+) cells according to cryopreservation time

Lioznov *et al*[17] reported that ALDH activity showed good correlation with the success rate of HCT, and we hypothesized a similar outcome with UCB. Thus, in this study we measured the relative numbers of high-level ALDH (+) cells among the lymphocytes, and confirmed ALDH (+) cells in all samples (Fig. 2). These ALDH (+) cells represented 0.78 ± 0.20% (range, 0.58–1.18%), 0.23 ± 0.07% (range, 0.12–0.39%), and 0.17 ± 0.09% (range, 0.08–0.40%), of the lymphocytes in the pre-freeze, 2-week, and 1-year cryopreserved samples, respectively. Thus, the

Table 1.

	TNC cells ($\times 10^8$ / unit)			TNC viability (%)			CD34+ cells ($\times 10^5$ / unit)			CD34+ cell viability (%)		
Cell Type	Ave	SD	*p*	Ave	SD	*p*	Ave	SD	*p*	Ave	SD	*p*
pre-freeze	5.1	3		98.2	1		9.6	7.3		97.8	0.7	
2 weeks cryopreservation	4.3	9	0.589	96.7	2.4	0.167	6.7	5.6	0.308	97.7	1.2	0.122
1 year cryopreservation	3.2	5.6	0.208	94.7	4.3	0.033	5.7	4.8	0.162	96.5	2.6	0.008

Analysis of the TNC counts, TNC viability, CD34+ cell counts and CD34+ cell viability in pre-freeze, 2-week and 1-year cryopreserved-thawed UCB samples (n = 10). TNC counts were determined with an automated hematology analyzer.
CD34+ cell enumeration was determined with a dual platform protocol and the gating strategy was based on ISHAGE guidelines. Viability assays for TNC and CD34+ cell were performed by acridine orange / propidium iodide dual staining and by flow cytometry.

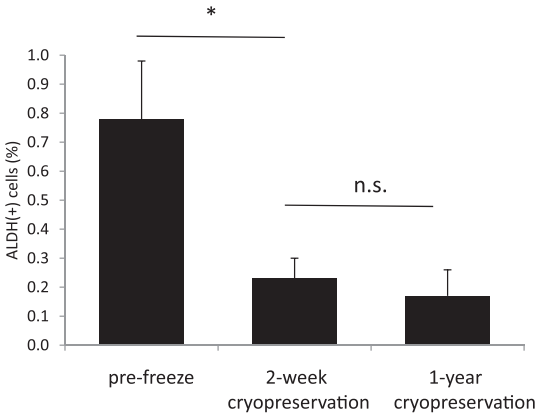

Fig. 2. The percentages of ALDH (+) cells from matched pre-freeze, 2-week, and 1-year cryopreserved samples. The percentages of high-level ALDH (+) cells among lymphocytes in pre-freeze, 2-week, and 1-year cryopreserved samples were measured by flow cytometry. $^*P<0.001$

number of ALDH (+) cells in the 2-week and 1-year cryopreserved samples were significantly decreased compared with those in the pre-freeze samples. There were no significant differences between the 2-week and 1-year cryopreserved samples.

Effects of cryopreservation on ALDH (+) cells and their subpopulations

The percentage of CD34+ and CD34+ CD38− cells did not reveal any significant changes at any point after cryopreservation compared with the pre-freeze cells (Fig. 3A and 3B). On the other hand, both the CD34+ and CD34+ CD38− cell possessing high levels of ALDH activity were dramatically decreased after 2 weeks and 1 year of cryopreservation compared with those in the pre-freeze samples. Moreover, prolonged cryopreservation produced further significant decreases in the CD34+ and CD34+ CD38− cells with high ALDH activity (Fig. 3C and 3D).

TNCs, CD34+ cells, and CFU-GM doses are generally measured in the cryopreserved UCB units prior to transplantation in order to characterize product quality. Here, the 2-week and 1-year cryopreserved samples showed decreased CFU-GM numbers compared with the matched pre-freeze samples, at plating levels of 2500 CD34+ cells (Fig. 4A). However, the length

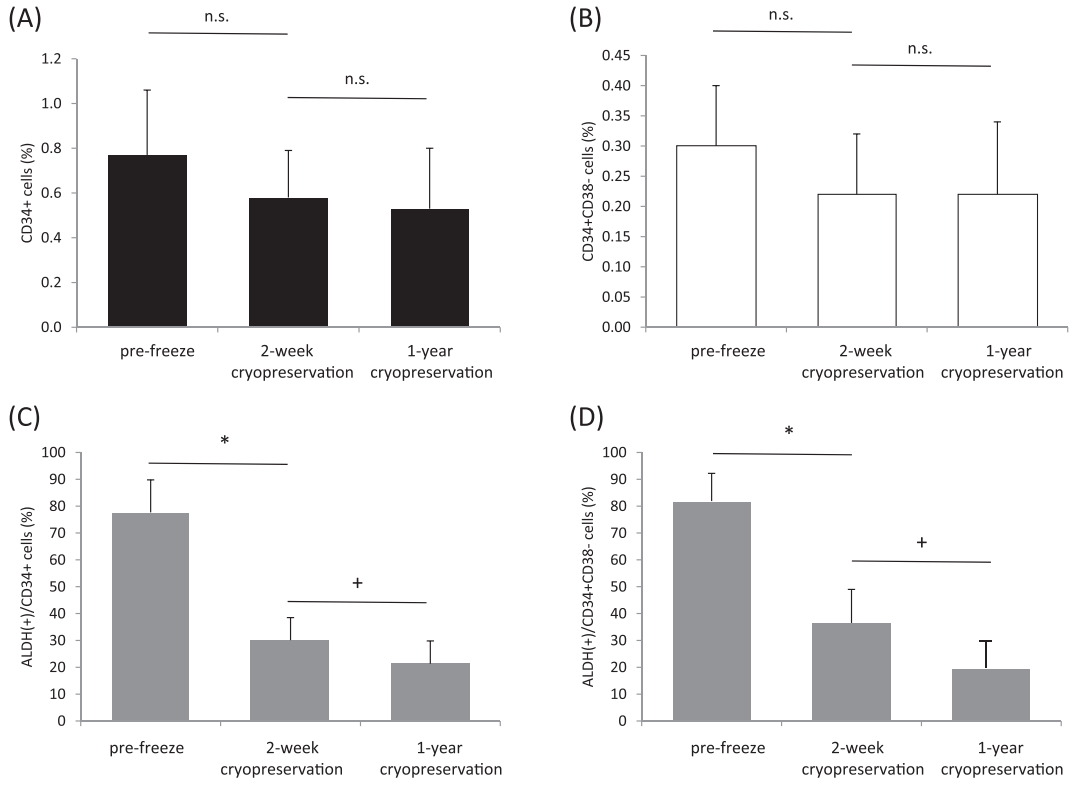

Fig. 3. Effects of cryopreservation on the percentages of CD34+ cells (A), CD34+ CD38− cells (B), ALDH (+) / CD34+ cells (C), and ALDH+ / CD34+ CD38− cells (D) among lymphocytes in UCB samples. All cell populations were stained with anti-CD34-PE antibody, anti-CD38-APC, and ALDEFLUOR, and then analyzed by flow cytometry. Negative controls were based on staining with isotype controls at each time point. Mean percentages of the matched 10 UCB samples in pre-freeze, 2-week and 1-year cryopreserved samples are shown. $^{*}P<0.001$, $+P<0.05$

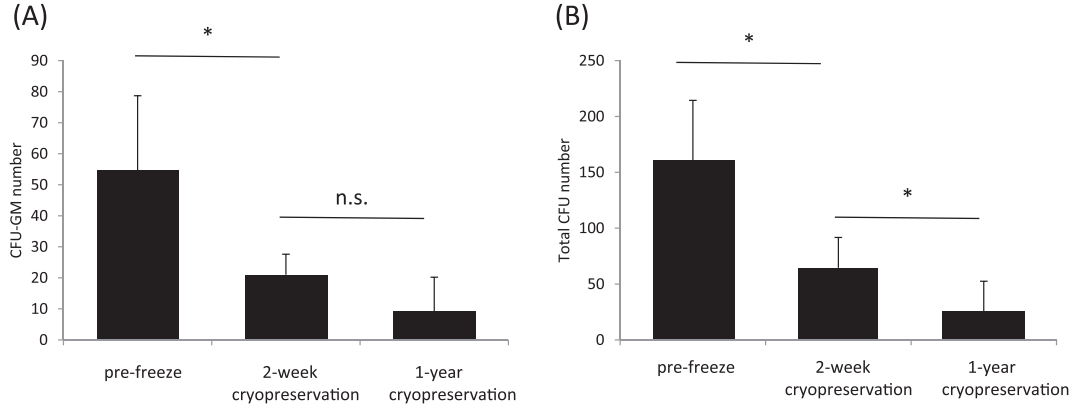

Fig. 4. The numbers of CFU-GM (A) and total CFU (B) per plate from 2500 isolated CD34+ cells. CD34+ cells were isolated on magnetic-activated cell sorting columns using microbead-conjugated antibodies. Sorted CD34+ cells were placed in 35-mm tissue culture plates with MethoCult GFH4434V. CFU-GM and total CFU were counted under an inverted microscope after 14 days. Mean values from the matched 10 pre-freeze, 2-week, and 1-year cryopreserved UCB samples are shown. $^{*}P<0.001$

of storage did not affect the viability of CFU-GM. Page *et al*[19] recently demonstrated that the total CFU number is a strong independent predictor of engraftment after unrelated UCB transplantation. Therefore, we also measured the total CFU numbers in all samples prepared for this study (Fig. 4B), and found a significant and continuous decrease over time with cryopreservation in the total CFU value.

Discussion

Cryopreservation-thawing does not affect the numbers of CD34+ or CD34+ CD38− cells, thus the number of TNCs, CD34+ cells, and CFU-GM are generally counted prior to HCT. However, several groups have reported that these counts and the success scores of transplantation do not always correlate[20, 21]. CD34+ cell numbers have been assessed with extrinsic markers, such as CD38 and CD133; however, these marker results are dependent on the antibodies used, staining methods, flow cytometry procedures, and other technical issues. Consequently, the CD34+ cell number might not be the best measure of hematopoietic activity for cryopreserved and banked UCB. ALDH is gaining popularity as another marker for evaluating early hematopoietic cells[12, 22]. The numbers of ALDH (+) cells in HSC grafts have been shown to correlate well with clinical outcomes after both autologous and allogeneic HCT as well as cryopreserved graft HCT[17, 23]. Indeed, Liozonv *et al*[17] showed a good correlation between engraftments and ALDH (+) cells, but not CD34+ cells or cell numbers, in fresh autologous peripheral blood stem cell transplants. However, very few papers have been published about the effects of ALDH (+) cell number in cryopreserved samples. Kurtz *et al*[24] reported that the median levels of CD34+ cells with high ALDH activity were 69 % before cryopreservation and 45 % after cryopreservation-thawing. Consistent with this, our data from the present study also indicated drastically decreased numbers of CD34+ / ALDH (+) cells and CD34+ CD38− / ALDH (+) cells after cryopreservation compared to numbers in pre-freezing. Together, these results suggest that measuring both CD34+ cell number and ALDH (+) cell number is a better way to assess the hematopoietic activity of cryopreserved UCB.

The CFU dose is also a strong independent predictor of engraftment after unrelated UCB transplantation; therefore, it should be used to assess potency when selecting UCB units for transplantation. In particular, measurement of the post-thaw CFU dose, or a more reliable surrogate, should be an important step during graft selection[18]. Our data showed that the numbers of CD34+ cells / total CFU and CD34+ cells / CFU-GM clearly decreased with cryopreservation, and that the CFU-GM number in particular was sensitive to prolonged frozen storage. This pattern of effect on CFU-GM was very similar to that for ALDH (+)/ CD34+ cells. On the other hand, since CD34+ and CD34+ / CD38− cell numbers did not change with regard to cryopreservation and the period of freezing, we suggest that there is a strong relationship between CFU-GM numbers and ALDH (+) / CD34+ cells, and we are investigating this relationship in detail in ongoing studies.

It is well known that determination of total CFU and CFU-GM is useful for assessing hematopoietic activity. However, the CFU assay is limited by its long readout times and issues

with standardization among centers. Flow cytometry is clearly a better way to measure the number of cryopreserved ALDH (+) cells because the method is simple and well established and thus suitable for use as a standardized measurement. We suggest assessing ALDH (+) cell numbers as an alternative to measuring total CFU or CFU-GM numbers prior to transplantation, and that this strategy could contribute to the selection of UCB from unrelated donors and improved transplantation results.

Conflict of interest

The authors have declared no conflict of interest.

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