

Long term cryostorage of UC blood units: ability of the integral segment to confirm both identity and hematopoietic potential

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Background

With the maturation of UC blood banking, cord blood (CB) units stored for years prior to use in transplantation present a new set of issues in clinical transplantation, including improvements in immune typing and confirmation of identity and viability. A preliminary analysis of the transplants supported by the St Louis Cord Blood bank, looking for an impact of length of CB storage time and transplant outcome was performed. We evaluated the utility of an integral segment containing an aliquot of cryopreserved product that has been exposed to the same post-processing storage conditions as the unit as a quality control tool for CB banking.

Methods

Engraftment and survival following unrelated donor UC blood transplant were evaluated based on length of CB product storage at the St Louis Cord Blood Bank. A strategy of routine testing of the contiguous segment for high-resolution HLA typing (also confirming identity) and CFU analysis was tested in 283 consecutive CB searches. Comparison between CB unit and contiguous segment viability and hematopoietic potential was performed on 30 research CB units that had been stored up to 5 years.

Introduction

UC blood is an alternative source of hematopoietic stem cells for both family members and unrelated donor transplantation [1–4]. Unique to cord blood (CB), compared with other hematopoietic stem-cell sources, is the practice of collecting, characterizing and storing CB units

Results

There was no statistical difference in engraftment or survival following unrelated donor cord blood transplant employing units banked < 1 year or > 3 years. Confirmatory HLA typing, CFU and viability testing was successfully performed from the same segment as part of a strategy for product release evaluation. When comparing the segment with its corresponding CB unit, the total colony-forming units (CFU) measured in the two was similar ($P = 0.51$, paired t -test). Three research units purposely sabotaged by an overnight thaw and refreeze had no CFU growth, but viability as measured by Trypan was still 68–98%.

Discussion

No deterioration of hematopoietic potential has been detected with storage up to 5 years. The contiguous segment CFU is representative of the product, and thus is a useful tool for quality control and confirmation of product viability. Viability, as measured by Trypan blue dye exclusion may be falsely reassuring.

Keywords

Cord blood, long-term cryopreservation, hematopoietic potential, contiguous segment.

for years prior to use. As CB banking enters its second decade, there are now units available that have been stored for many years — a situation rarely encountered in BM or peripheral blood transplantation.

There are reports of BM products cryopreserved for up to 11 years that have resulted in hematopoietic recovery

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when transplanted, but most products are used within 1 year of collection [5,6]. In the laboratory, CB units stored > 10 years have been demonstrated to contain viable hematopoietic progenitors based on *in vitro* assays [7,8]. While long-term cryopreservation of CB hematopoietic precursors is possible, it is important that CB banks be able to confirm that a given unit being considered for transplantation still has viable hematopoietic progenitors. There is a theoretical increase in risk of cellular deterioration with long-term storage. Additionally, the loss of institutional memory that comes from personnel and technology changes is a concern. The risk of purposeful or accidental thawing/refreezing and transient warming events is real, especially in banks where inventory has been exposed to conditions outside of liquid nitrogen.

The practice at the St Louis Cord Blood Bank has been to routinely heat-seal a sample of CB in a segment of contiguous tubing prior to cryopreservation of the unit. This integral CB sample is exposed to the same freezing and storage conditions as the unit. Using the contiguous segment is a concept transferred from the routine blood banking practice of using the attached tubing from a whole blood unit to crossmatch or confirm ABO/Rh. As part of the routine search and product release procedures at the St Louis Cord Blood Bank, high resolution molecular HLA typing is repeated on an integral cord sample prior to release of product for transplantation. This both confirms product identity and updates HLA typing. In our initial experience with over 2000 direct searches and 300 product releases, there have been several instances where the confirmatory testing did not corroborate the initial HLA report. These were primarily cases of serologic interpretation errors and of new allele detection as methods of HLA typing have improved. However, transcription errors in HLA reporting have been found. The use of the contiguous sample, rather than samples that are stored in separate aliquots — usually in separate freezers — eliminates the small risk of product mislabeling or misplacement. The contiguous segment is representative of the product that is being considered for clinical use, especially as the cells within the contiguous segment have experienced the same processing, freezing and cryostorage conditions as the whole CB unit.

The purpose of this study was to analyze contiguous segments to determine if it was possible to confirm HLA typing, and to assess the hematopoietic potential and viability of cryopreserved units. If so, this would be an

important quality assurance tool. Additionally, we evaluated the engraftment kinetics and survival for transplants supported with CB units from the bank — particularly with respect to the impact of length of storage on transplant outcome.

Material and methods

St Louis Cord Blood Bank program

Umbilical CB was collected as part of the First Gift Program at the St Louis Cord Blood Bank. In this program, regional obstetricians/midwives and obstetrical units collected CB during the third stage of labor from volunteer families under an Institutional Review Board (IRB) approved trial and regulated by the FDA as an Investigational New Drug (IND). Umbilical CB is collected into a 250 mL blood collection bag containing citrate phosphate dextrose (CPD) anticoagulant as previously described [9]. Basic demographic and transplant outcome data was provided by participating transplant programs that received products from the bank.

Laboratory processing and cryopreservation

CB units were processed within 36 h of collection. All CB units were RBC and plasma depleted before addition of a 50% DMSO/Dextran freezing cocktail (final concentration of DMSO was 10%), and then stored in liquid nitrogen following a controlled rate freeze — as reported in our previous studies [10]. The segment is formed from the tubing through which the CB enters the cryobag, composed of same plastic as the cryobag (Baxter Cryocyte freezing container with label pocket 50 mL and 250 mL). Cord blood cells remain in this small section of tubing (approximately 4 cm long).

Contiguous segment

Segment thaw, colony-forming unit assay and viability

The cryopreserved segment was detached from the unit in vapor phase of liquid nitrogen and immediately thawed in a 37°C waterbath. Contents of the segment were removed with a tuberculin syringe (1 cm³, 28.5 ga, Becton Dickinson). One drop (approximately 4μL) of the thawed cell suspension was added to 3 mL of methylcellulose media and cultured as described below. The number of cells inoculated was calculated from the post-processing total nucleated cell concentration, corrected for added volume of cryopreservation cocktail. A second drop (4 μL) of cell suspension was diluted into 0.5 mL of Dextran for Trypan

viability assessment. The remaining volume of blood was applied to filter paper (Schleicher & Schuell) for confirmatory molecular HLA typing. HLA typing was performed by Laboratory Corporation of America using a PCR/sequence specific oligonucleotide probes (SSOP) technique.

Standard thaw

The corresponding 30 CB units were removed from liquid nitrogen and immediately thawed in a 37°C waterbath following the thaw procedure developed by Rubinstein *et al.* [11]. Briefly, an equal volume of 10% Dextran/5% albumin was added to the thawed product. Units were spun at 400 g for 10 min at 10°C. After centrifugation the supernatant was expressed. The product was then gently resuspended and a sample removed for WBCC, colony-forming unit (CFU) quantitation, Trypan blue and 7AAD viability, determined by flow cytometry.

CFU assay

Nucleated cells were cultured in duplicate using a semi-solid clonogenic assay [10]. For the CB unit, 2×10^5 cells were inoculated into 3.0 mL of methylcellulose media (Methocult, Stem Cell Technologies) and equal volumes plated into two wells (6-well, Falcon). For the segments, one drop (approximately 4 μ L) was added to 3 mL media and plated in duplicate as above: the actual number of cells inoculated was calculated from the concentration of nucleated cells within the thawed unit, corrected for the freeze cocktail. The plates were incubated at 37°C in 5% CO₂ humidified incubator. At 14 days, CFU-granulocyte-macrophage (GM), CFU-Mix and blast-forming units erythrocyte (BFU-E) were scored using an inverted microscope.

Viability measured by Trypan blue

A single drop of cell suspension was diluted into 0.5 mL of Dextran for Trypan viability assessment. The RBC were lysed with a 4% *tris*-ammonium chloride solution and then Trypan blue stain (0.4%, Gibco) was applied. The Trypan blue cell suspension was incubated for 5 min at room temperature and then the nucleated cell viability was evaluated using a Neubauer hemacytometer and light microscope.

Viability measured by 7-amino actinomycin D

CB nucleated cells were triple labeled with the 7AAD (7-amino actinomycin D, Beckman Coulter) viability dye, the anti-CD45 MAb directly conjugated with FITC and the anti-CD34 MAb directly conjugated with PE using Beckman Coulter's Stem-Kit CD34⁺ HPC Enumeration Kit following the manufacturer's protocol. An Isotype PE control was performed in parallel. Flow cytometric analysis was performed using the Coulter Epics XL. Seventy-five thousand events were analyzed for each assay.

Sabotaged units

To mimic purposeful or accidental thaw, three research units were removed from liquid nitrogen and allowed to thaw overnight (approximately 16 h) at room temperature. The units were then plunged back into liquid nitrogen for 1 h and thawed following the standard unit thaw procedure described above. The units were assessed for CFU potential, Trypan blue viability and viability by 7AAD.

Cell counts

The SYSMEX SE-9500 or XE-2100 hematology analyzers were used to count total nucleated cells, including nucleated RBCs.

Statistics

Univariate analysis of neutrophil recovery and survival following CB transplantation was calculated using the method of Kaplan and Meier, and was compared using the log rank statistic. A paired *t*-test was used to compare the viability and CFU data from the segment to its corresponding unit. $P < 0.05$ was considered to be significant. All statistical analyses were assessed with software from the Statistical Package for the Social Sciences (SPSS, Chicago).

Results

Lack of impact of storage time on engraftment or survival following CB transplant

Using data collected from units exported by the St Louis Cord Blood Bank, engraftment (as defined by absolute neutrophil recovery $> 500/\mu$ L on 3 consecutive days) and survival (to Year 1) were evaluated based on length of time of CB storage (Figures 1 and 2). There was no difference statistically in neutrophil recovery or 1-year survival when CB units were in cryostorage > 3 years compared to those stored < 1 year.

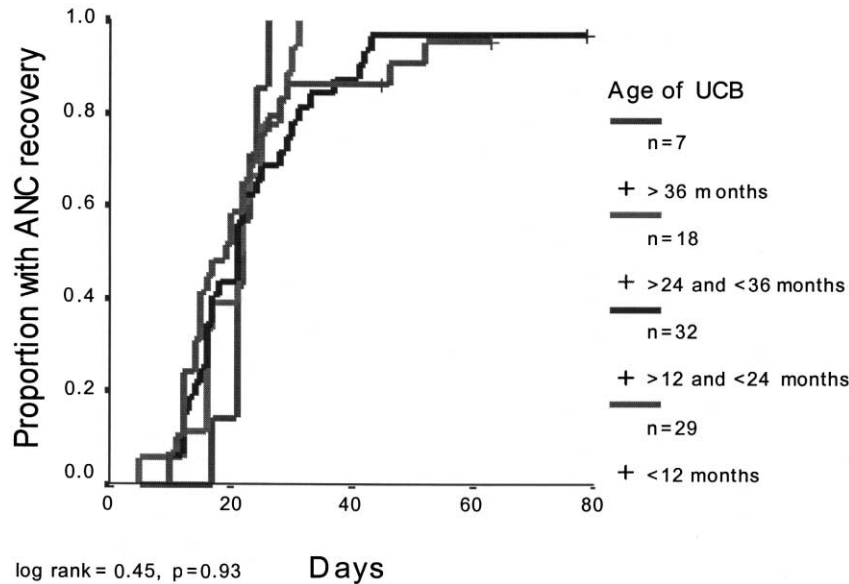


Figure 1. Lack of impact of length of storage of CB unit on neutrophil recovery following unrelated donor CB transplant.

Segment contains sufficient material both for HLA confirmation, CFU quantitation and Trypan viability analysis

Two hundred fifty six (256) CB unit segments were studied at time of evaluation for potential use in transplantation. Two hundred and fifty three (253) of the samples were evaluable for both CFU and molecular HLA typing. The three nonevaluable samples became contaminated during the CFU assay incubation. All were evaluable

for HLA typing. The mean segment CFU was 55 ± 36 colonies/ 10^5 cells (range: 0.3–197). The mean Trypan blue viability was $98 \pm 2\%$ (range: 80–100).

Segment CFU can be used to represent the hematopoietic potential of the thawed CB unit

Thirty research CB units and their contiguous segments were thawed and evaluated for viability and CFU content. The length of storage of the units ranged from 32 days to

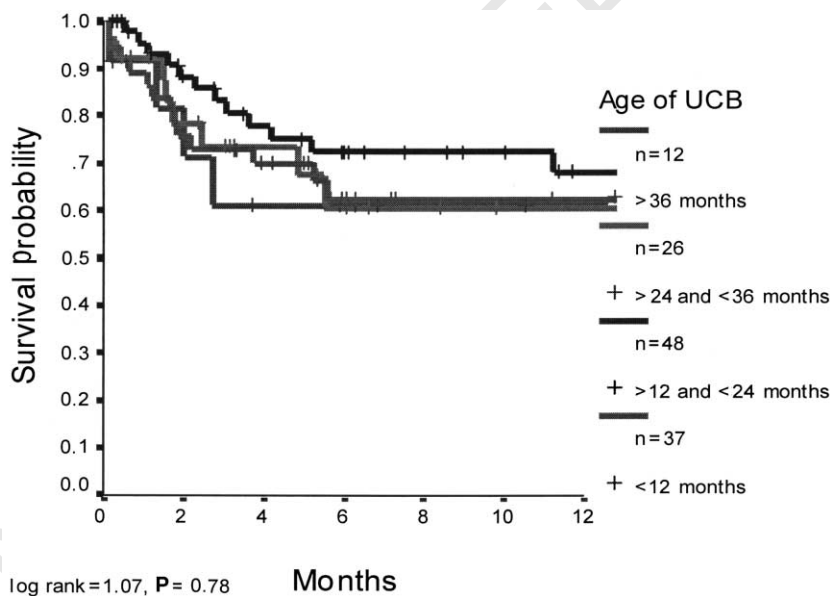


Figure 2. Lack of impact of length of CB unit storage on survival following unrelated donor CB transplant.

Table 1. CB units compared with their contiguous segments: CFU and viability

	Segment n = 30	Unit post- thaw n = 30	P
CFU assay:			
Mean colonies/ 10^5 cells \pm SD	49 \pm 38.1	59 \pm 31.2	0.51
(range)	(5–145)	(7–141)	
Trypan blue viability (%):			
Mean \pm SD	98 \pm 1.6	97 \pm 7.9	< 0.002
(range)	(92–99)	(71–99)	

Note: Dataset does not include any of the sabotage units.

Trypan blue viability is a poor measure of product HPC viability in sabotage setting (following freeze-thaw)

Three units were treated in a simulated product sabotage. The sabotaged CB units were removed from liquid nitrogen, stored at room temperature overnight and then plunged back into liquid nitrogen. One hour after re-freeze, the units were thawed per laboratory protocol and the viability of product and segment were compared. There was no CFU growth in either segment or CB unit (Table 2). However, the Trypan blue viability of the nucleated cells was 68–98%. When the alternative viability stain, 7AAD, was used, the viability of the nucleated cells in the sabotaged units was 18–20% (normal post-thaw 7AAD viability ranges 32–77%).

Discussion

One of the major advantages of CB is that it is banked, characterized and rapidly available for transplantation. As CB banking enters its second decade, the length of cryostorage becomes a significant issue. The risk that the product may naturally lose potency during liquid nitrogen storage, be exposed to temperatures outside the recommended storage range, or may be accidentally thawed is small but should be anticipated. Additionally, over time laboratory practices and personnel evolve. It is important

5.2 years. There was a strong relationship between CFU content in the segment and the unit (Table 1). Figure 3 compares the HPC content of the segment with its corresponding unit. In five cases out of 30 there was > 50% difference between the segment and unit; in three cases the segment over-estimated the CB unit and in two cases the segment under-estimated CB unit. In all instances CFU were present — regardless of length of storage.

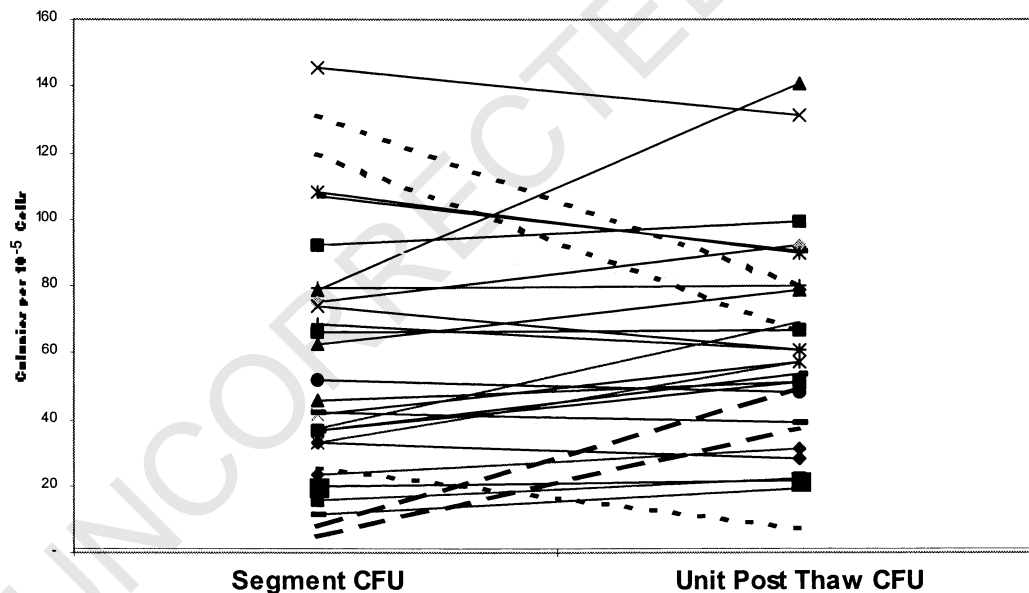


Figure 3. Relationship between thawed unit and contiguous segment.

Table 2. CFU and Trypan blue viability before and after 'sabotage' (thaw–refreeze)

No.	Trypan blue viability (%)		CFU colonies/10 ⁵ cells		7AAD viability (%)
	Post-processing ¹	Post-sabotage ²	Post-processing ¹	Post-sabotage ²	Post-sabotage ²
1	98	68	113	0	20
2	97	98	45	0	19
3	95	94	101	0	18

¹After processing but before cryopreservation.

²Post-thaw.

that CB banks develop product validation and quality control measures that confirm both product identity and viability. The application of a standard blood-banking tool — the analysis of a contiguous segment — offers the ability to confirm product identity, as well as provide information about the HPC capacity of the CB unit. Thus far, no delay in engraftment or decrease in survival following CB transplant has been identified based on length of storage for periods up to 3 years. It will be important to reevaluate this issue over the years as banked inventories age.

This study has demonstrated that the CFU content of the contiguous segment reflects that of the corresponding thawed CB unit. In our controlled setting, with extrapolation for small test volumes, we found excellent correlation in CFU content between CB segment and product. Since there is considerable variability with CFU as an assay for hematopoiesis, the clinical utility is realistically limited to confirming the presence of hematopoietic precursors within the product, and not as a quantitative measure of hematopoietic potential. Nevertheless, as a tool for quality control, the presence of CFU in the segment supports the fact that the unit contains viable hematopoietic progenitors within the product intended for transplantation. Furthermore, it is feasible to perform CFU, Trypan blue viability and confirmatory HLA typing from the integral segment prior to product release.

The described sabotage scenario represents one situation where a product would otherwise escape detection on visual inspection prior to release. Less dramatic is the impact of repeated transient warming and other unexpected events. The risk of such events (albeit small) can be expected to increase as products are stored for longer periods of time.

It is worrisome that Trypan blue viability can remain high in a setting where there are no viable hematopoietic precursors based on CFU activity. Other viability stains, such as propidium iodide or 7AAD, may be more useful and need to be studied. Current studies are ongoing using 7AAD to assess viability in CB units post-thaw. At issue is that cells post-thaw may have membrane leakiness that may give false positive or negative results.

There are limitations with the use of the integral segment. Since, there are a finite number of segments attached to a given CB unit, the product can only be tested once or twice prior to use. This is adequate for confirmatory HLA testing, but limits viability testing. Frequently, multiple CB units are evaluated for any given patient. This results in some units being considered up to 10 times before being selected for use in transplantation. Additionally, the CFU assay requires 14 days and there are times when the CB unit is selected for transplant prior to completion of the CFU assay. Given the length of preparative regimens is usually 7 days, in most instances the CFU results would be available prior to infusion. Lastly, caution should be used in interpreting the segment CFU results and their relationship to the CB unit. The segment is simply an indicator of the hematopoietic potential of its corresponding unit, and its values should not be construed as the exact colony content of the thawed unit. We recommend consideration of a pre-transplant CB product-release evaluation that includes:

- A confirmation of product identity
- A measure of hematopoietic potential.

The use of the contiguous segment offers both. This will provide the clinician with confirmation that the CB product that was banked a decade ago truly is the product

that they believe they are getting, and that it still contains viable hematopoietic progenitors.

Acknowledgements

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