Multilineage engraftment of refrozen cord blood hematopoietic progenitors in NOD/SCID mice

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Seven cord blood (CB) units were tested for their capacity to repopulate irradiated NOD/SCID mice after one or two successive cryopreservation procedures. In primary transplants with frozen or refrozen CB cells we observed equivalent human colonies and percentages of human CD45+ cells, with multilineage engraftment. In secondary transplants flow cytometry and polymerase chain reaction for the α satellite region of chromosome 17 showed equivalent levels of human engraftment. Since CB units have, to date, mainly been stored in individual bags, our results suggest new options for optimizing the timing of infusions of expanded and non-expanded progenitors in transplants.

Key words: cord blood transplantation, cryopreservation, refreezing.

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Cord blood (CB), a source of hematopoietic stem cells (HSC) with a high proliferative and repopulating capacity, can be successfully transplanted for the treatment of various hematologic and non-hematologic diseases.[1,2] Even though CB HSC have some intrinsic biological advantages over bone marrow or mobilized peripheral blood HSC,[3,4] their number in a single CB unit is limited. Delayed neutrophil and platelet engraftment and a high incidence of graft failure have been observed in pediatric patients and adults transplanted with low doses of CB HSC.[5-7] *Ex vivo* expansion of HSC might circumvent this problem.[8] As most units in CB banks are stored in individual bags, one approach has been to thaw the single unit, infuse the non-expanded component at time 0 and then infuse *ex vivo* expanded progenitors 10-12 days later.[9,10] However, this procedure does not accelerate the early engraftment.[11] A better alternative might be to cryopreserve CB in multiple bags in order to infuse expanded and unexpanded cells simultaneously.[12] We previously demonstrated that two successive freeze-thaw procedures do not significantly affect either the clonogenic potential or cell adhesion molecule expression of CB progenitors.[13] This suggests that when CB units are stored in a single bag it would be possible to thaw the single unit, divide it into two unequal parts, expand the smaller part, refreeze the other one and then infuse the expanded and unexpanded progenitors at the same time to accelerate early engraftment. Here, we have investigated the possibility of engrafting NOD/SCID mice with CB HSC which underwent two successive cryopreservation procedures.

Design and Methods

CB collection and processing

Collection and cryopreservation procedures were performed according to an ISO 9002 quality system validation program of the Italian Cord Blood Banks Network.[14] Informed consent was given. Seven CB units obtained after delivery of full-term neonates were collected in MacoPharma bags (Rho, Milan, Italy) containing 21 mL citrate phosphate dextrose (CPD). Total volumes (including the anticoagulant) ranged from 65 to 127 mL (mean 83±20 mL). From each of four CB units, 20 mL of blood were utilized for clonogenic tests, flow cytometry analysis and primary transplants, before cryopreservation. The units were then transferred with no prior cell separation into 200 mL Haemofreeze DF700 bags (NPBI, Emmer-Compascuum, Netherlands) which, after adding the sterile saline solution plus 5% human albumin, 10% DMSO and 2000 I.U. heparin to make a final volume of 200 mL, were cryopreserved by a standard method (-1 °C/min. programmed cooling rate, stored in liquid nitrogen). Storage before the first thawing lasted from one to two months. The CB units were thawed at 37°C, gently mixed in melting ice and quickly refrozen within 15 min. A 45 mL sample was withdrawn, slowly mixed with 45 mL of sterile washing solution (5% Dextran 40, 2.5% human albumin in 0.9% NaCl) and centrifuged once (400 g/10 min.). The supernatant was removed and the sedimented cells were slowly resuspended in fresh washing solution which was added to restore the volume to 45 mL, then 1.5 mL were tested for viability, burst forming units-erythroid (BFU-E), colony forming units-granulocyte-macrophage (CFU-GM), colony forming units-mixed (CFU-MIX) and flow cytometry and 43.5 mL were centrifuged over Ficoll-Hypaque (1.077 sd, Pharmacia, Uppsala, Sweden). Low density mononuclear cells were used for assays of colony forming units-megakaryocyte (CFU-Mk), and long-term culture-initiating cell (LTC-IC)

as well as for the mice transplants. This procedure was repeated twice with intervals of one month or more between freezing and thawing.

Cell cultures

Assays for BFU-E, CFU-MIX, and CFU-GM were performed[13] in methylcellulose medium with human recombinant cytokines Methocult GF H4434 (StemCell Technologies, Vancouver, Canada). CFU-Mk were assessed by the MegaCult™ assay (StemCell Technologies), according to the manufacturer's instructions. LTC-IC assays were performed[13] in MyeloCult H5100 medium (StemCell Technologies). All cultures were set up in triplicate.

Flow cytometry of CB samples

Whole CB and thawed samples were analyzed by a three-color technique for: CD34/CD38/ CD13 and CD34/CD45/annexin. Fluorescein isothiocyanate conjugated annexin V was from R&D Systems (Minneapolis, USA). Nucleated cells (5×10^5) were incubated for 20 min at 4°C with the monoclonal antibodies. After incubation and red cell lysis by ammonium chloride, the cells were analyzed with an XL2 EPICS COULTER cytometer equipped with an argon laser. Almost 200 $CD34^+$ cells were analyzed for each sample. Absolute $CD34^+$ counts were assessed by a two-platform ISHAGE-derived method.[13]

Animals

NOD/LtSz scid/scid (NOD/SCID) mice (from Jackson Laboratories through Charles River Italia [Calco, Italy]) were maintained in the animal facilities at the CIOS. Immunogenetic and Experimental Oncology Center, Turin, Italy. Forty-five mice (two or three for each experimental condition: Basal, Thaw1, Thaw2) were utilized for primary transplants. The mice were irradiated at 6 to 8 weeks of age with 350 cGy of total body irradiation from a ^{137}Cs source and, after 24 hours, were given a single intravenous injection of 10×10^6 low density mononuclear cells.[15] They were killed 5 weeks after transplantation and bone marrow from the femora and tibiae was analyzed for human engraftment.

Evaluation of human engraftment by flow cytometry and cell cultures

In order to perform the two or three-color analysis, 1×10^6 mouse bone marrow cells, previously incubated with human and mouse immunoglobulins (Sigma, St Louis, USA), were incubated with anti-mouse CD45/anti-human CD45 or with the following anti-human monoclonal antibodies: anti-CD45/CD34, CD45/CD33, CD45/CD19, CD45/CD61/CD34, CD45/71/glyA, and CD45/38/34. Bone marrow cells from sacrificed mice were also used to perform BFU-E, CFU-GM, CFU-MIX, CFU-Mk and LTC-IC assays using human recombinant cytokines (species-specific), as described above, to obtain a functional evaluation of human engrafted cells.

Secondary transplants

Secondary transplants were performed for two of the CB units: 40×10^6 low density mononuclear cells from the bone marrow of primary transplanted mice were injected in secondary recipients. For every CB unit, four secondary mice were transplanted, two for each experimental condition (one or two freeze-thaw procedures: total eight mice). Secondary recipient mice were then sacrificed after 5 weeks and human engraftment was investigated by evaluating human CD45 expression in the bone marrow and by molecular analysis for the α-satellite region of chromosome 17 in the peripheral blood bone marrow and spleen.

DNA extraction and molecular analysis of human cell engraftment in secondary recipient mice

High-molecular weight DNA was extracted from the bone marrow, peripheral blood, and spleen of each mouse by the NucleoSpin Blood Kit (Machery-Nagel Inc. Easton, PA, USA). The presence of human-specific DNA within the murine bone marrow of transplanted mice was confirmed by polymerase chain reaction (PCR) amplification of an 850-bp fragment of the α-satellite region of human chromosome 17 using the forward primer: 5'GGGATAATTTCAGCTGACTAAA and the reverse primer: 5'TTCCGTTTAGTTAGGTGCAGTTATC. The reaction was performed in a final volume of 50 μL using 0.5U of Taq DNA polymerase (Fermentas Inc. Hanover, MD, USA), 250 nM of each primer, 200 μM of each nucleotide, and 2 mM $MgCl_2$, in 1x Taq buffer containing $(NH_4)_2$ SO_4. Following an initial DNA denaturation at 94°C for 10 min, 35 1-min cycles of denaturation at 94°C, annealing at 60°C and extension at 72°C were performed before a final elongation step at 72°C for 10 min. All amplified DNA fragments were stained with ethidium bromide, electrophoresed through 1% agarose gel and visualized in ultraviolet light. As an internal control, the presence of the glyceraldehyde phosphate dehydrogenase (GAPDH) house-keeping gene was analyzed using the forward primer: 5'ACCACAGTCCATGCCATCCAC and the reverse primer: 5'TCCACCACCCTGTTGCTGTAG which amplified a 555 bp fragment of genomic DNA of both mouse and human origin. The amplification occurred after 5 minutes at 94°C, 35 cycles of 1 min at 94°C, 1 min 30 seconds at 60°C, 1 min 30 seconds at 72°C and a final step at 72°C for 10 min.

Statistical analysis

SCID engrafting potential was defined as the total number of human cells per mouse/number of cells transplanted. The Student's t test and Wilcoxon's test were used for the statistical analysis.

Results and Discussion

The percentage of annexin V positive $CD34^+$ cells increased from the basal value (before cryopreservation) of 3.6±1.7 to 9.2±4.3 after the first thawing (p=0.051) and to 11.1±3.6 after the second thawing (Thaw1 versus Thaw2, p=0.17; basal versus Thaw2;

Table 1. Flow cytometric analysis of CD45+ human subpopulations in 34 primary NOD-SCID mice transplanted with low density mononuclear cells from seven CB units after one (Thaw 1) or two (Thaw 2) successive freeze-thaw procedures shows multilineage engraftment.

	huCD45+%	*CD34+%*	*CD34+/38-%*	*CD34+/38+%*	*CD34-/38+%*	*CD33+%*	*CD34+/61+%*	*CD71+/GlyA+%*	*CD19+%*
Basal	24.4±6.3	2.7±0.7	0.41±0.2	2.3±0.5	16.7±3.9	16.9±4.3	0.4±0.09	2.8±1.0	3.8±1.1
Thaw 1	25.4±5.9	2.6±0.6	0.03±0.01	3.1±0.6	20.1±4.7	16.1±3.9	0.4±0.07	2.4±1.1	5.4±1.2
Thaw 2	32.5±5.5	3.2±0.5	0.06±0.02	3.4±0.6	25.4±3.8	21.3±4.0	0.4±0.06	2.6±0.8	6.7±1.2

Basal: analysis in 11 mice transplanted with low density mononuclear cells from four out of seven CB units, before cryopreservation. Values are expressed as mean percentages of total nucleated cells (murine plus human) ± SE. Monoclonal antibodies: anti-CD34 PE (8G12, Becton-Dickinson, San Jose, CA, USA), anti-human CD45 FITC (2D1,BD), anti-human CD45 PerCP (2D1,BD), anti-mouse CD45 CY-CHROME (30-F11, PharMingen, San Diego, USA), anti-CD38 TC (HIT 2, Caltag, South San Francisco, CA, USA), anti-CD19 PE (SJ25-C1, Caltag), anti-CD33 PE (P67.6, BD), anti-CD61 FITC (RUU-PL 7F12, BD), anti-CD 71 FITC (L01.1, BD), anti-glycophorin A PE (JC159, DAKO, Glostrup, Denmark). The appropriate isotype control was performed.

Table 2. Human colonies in 34 primary NOD-SCID mice transplanted with LDMNCs from 7 CB after 1 (Thaw 1) or 2 (Thaw 2) successive freeze-thaw procedures.

	BFU-E	*CFU-GM*	*CFU-MIX*	*CFU-Mk*	*LTC-IC*
Basal	16.6±5.5	16.4±3.6	4.8±1.5	5.4±0.6	3.6±0.4
Thaw 1	29.2±6.4	26.8±4.0	7.4±1.5	4.6±0.7	1.8±0.5
Thaw 2	25.1±4.5	32.6±4.4	7.1±1.8	5.2±1.1	2.3±0.5

Basal: analysis in 11 mice transplanted with low density mononuclear cells from four out of seven CB units, before cryopreservation. CFU, BFU-E and LTC-IC are expressed per 10^5 mononuclear cells.

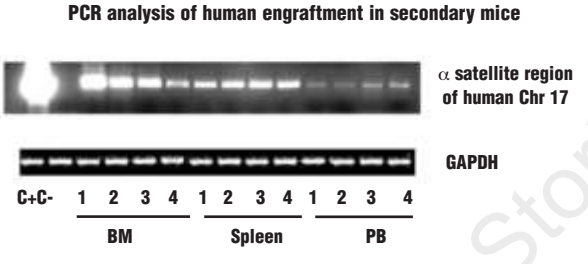

Figure 1. Representative PCR analysis of human engraftment in secondary recipient mice performed in bone marrow (BM), spleen and peripheral blood (PB) mononuclear cells. Four mice were analyzed, two for each experimental condition (one or two freeze-thaw procedures) Chr 17= chromosome 17: C+= positive control, C-= negative control.

p=0.03). The recovery of white blood cells, lymphocytes, CD34+ cells, CD34+/CD38-/CD13- cell subset, short-term and long-term colonies did not differ significantly between samples tested after one or two cryopreservation procedures (*data not shown*).

The percentage of human CD45+ cells measured in the bone marrow of NOD/SCID mice 5 weeks after transplantation was equivalent whether the graft had been thawed once (25.4±5.9), or twice (32.5±5.5), and was associated with multilineage engraftment in both experimental situations (Table 1). The mean percentage of human CD45+ cells in the mice transplanted with CB progenitors before cryopreservation (experiment performed with only four CB units) was 24.4±6.3. The SCID engrafting potential of CB low density mononuclear cells after the first and second cryopreservation procedures was 2.1±0.7 and 2.0±0.6, respectively, whereas the engrafting potential prior to cryopreservation was 1.5±0.6.

The clonogenicity of human BFU-E, CFU-GM, CFU-MIX, CFU-MK and LTC-IC from the bone marrow of primary recipient mice did not differ significantly according to whether the graft had been thawed once or twice (Table 2). Statistics performed by parametric (Student's t test) and non-parametric (Wilcoxon's test) methods provided equivalent results. To assess the long-term repopulating capacity of thawed cells, a secondary transplant procedure was performed. The bone marrow of mice transplanted with CB HSC that had undergone one or two cryopreservation procedures showed low, but equivalent, levels of human CD45+ cells (huCD45+ 1.9±0.1 and 1.9±0.2%, respectively). PCR analysis for DNA of the α-satellite region of human chromosome 17 performed on secondary recipient mouse peripheral blood, bone marrow and spleens confirmed the human engraftment after one and two cryopreservation procedures (Figure 1). PCR detection of the GAPDH house-keeping gene confirmed that the same amount of DNA was amplified in the above described PCR experiments. The resistance of relatively undifferentiated cells to refreezing has been described for human blastocysts which led to a successful pregnancy after two successive cryopreservation procedures.[16] Two successive freeze-thaw procedures do not significantly affect either the clonogenic potential or cell adhesion molecule expression of CB progenitors.[13] Further evidence of the resistance of CB cells to repeated cryopreservation derives from the report that CB NK cells can be expanded *ex vivo* after two successive freeze-thaw cycles.[17] In this study we first evaluated the sample quantitatively and qualitatively by flow cytometry and cell cultures. The absolute count of CD34+ cells and of the immature CD34+/CD38-/CD13- subset, as well as short and long-term colonies were not significantly affected by

refreezing. The percentage of apoptotic CD34$^+$ annexin V$^+$ cells increased slightly after each freeze-thaw cycle; however, the difference between the first and the second cycle was not significant. The percentage of human engraftment (evaluated by human CD45 expression) was similar in mice transplanted with frozen or refrozen CB low density mononuclear cells. SCID engraftment potential did not differ significantly either. Expression analysis of lineage-specific markers showed complete engraftment with myeloid (CD33$^+$), erythroid (glycophorinA$^+$ CD71$^+$), megakaryocytic (CD61$^+$), and lymphoid (CD19$^+$) commitment in both experimental conditions. Human short and long-term colonies from the bone marrow of NOD/SCID primary recipient mice did not differ significantly according to whether the recipients were injected with frozen or refrozen CB mononuclear cells.

The secondary transplants in mice showed less human engraftment (evaluated by human CD45$^+$ expression) than they had in previous studies performed with purified expanded or non-expanded CB CD34$^+$.[15] Nevertheless, we found no significant differences in the percentage of huCD45$^+$ cells among secondary recipients injected with frozen or refrozen CB cells. The study of human chromosome 17 α-satellite region DNA confirmed human engraftment in the peripheral blood, bone marrow, and spleen of secondary recipient mice injected with frozen and refrozen CB cells. In conclusion, our study demonstrates that two successive cryopreservation procedures do not significantly affect the ability of human CB HSC to produce long term engraftment in NOD/SCID mice. These results suggest that new approaches could be taken to in *ex-vivo* expanded CB transplant. With CB units cryopreserved in single bags, refreezing might allow the simultaneous infusion of expanded (after one freeze-thaw cycle) and non-expanded (after two freeze-thaw cycles) CB progenitors. This might accelerate early engraftment without losing the potential of the non-expanded cells to provide long-term engraftment.

FT conceived and designed the study, supervised flow cytometry and cell culture experiments, analyzed the data, and wrote the paper. NC performed flow cytometry and cell culture experiments and analyzed the data. AD, LF, MB performed flow cytometry and cell culture experiments. FS performed mouse transplants. LF performed cryopreservation procedures. YP performed PCR analyses. WP supervised in vivo experiments, interpreted the data and revised critically revised the article. EM and LCdM interpretated the data and critically revised the article. FF designed the study,supervised flow cytometry and cell culture experiments and wrote the paper. All the co-authors actively partecipated in the preparation of the manuscript and approved its final version. The authors declare that they have no potential conflicts of interest.

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