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The Effect of Cryopreservation on Clonogenic Capacity and In Vitro Expansion Potential of Umbilical Cord Blood Progenitor Cells

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ABSTRACT

Background. Umbilical cord blood progenitor cells have been demonstrated to possess significant advantages over bone marrow in terms of proliferative capacity and immunologic reactivity. But the low number of hematopoeitic stem cells (HSC) is the most important limitation of its use. The ex vivo expansion of cord blood progenitor cells is the current strategy to overcome this problem. Futhermore, among the factors that enable successful cord blood transplantation is the ability to store and subsequently recover a sufficient number of viable cells. Since it would be costly to expand umbilical cord blood (UCB) progenitor cells, it is important to determine the feasibility and reproducibility of progenitor cell expansion after cryopreservation. We evaluated whether cryopreservation procedures might impair the clonogenic capacity and in vitro expansion of UCB.

Materials and methods. We evaluated the cell viability, clonogenic capacity, $CD34^{+}38^{-}$ content and in vitro expansion potential of progenitor cells from UCB ($n = 10$) separated mononuclear cells (MNC), before and after 1 month of cryopreservation by programmed rate freezing.

Results. Although cell viability decreased after cryopreservation ($P < .05$), there was no significant difference in $CD34^{+}$ or $CD34^{+}38^{-}$ absolute count, colonogenic capacity and in vitro expansion potential of cord blood progenitor cells ($P > .05$).

Conclusions. Since the survival of $CD34^{+}$ cells was greater than other elements, $CD34^{+}$ cells seem more tolerant to cryopreservation than the other nucleated populations. Moreover in vitro expansion of UCB progenitor cells may be obtained following cryopreservation. Our results suggest that cryopreservation procedures do not impair the clonogenic capacity and in vitro expansion potential of cord blood stem/progenitor cells.

UMBILICAL CORD blood (UCB) has been recently considered an attractive potential alternative to bone marrow (BM) as a source of hematopoeitic progenitor cells ($CD34^{+}CD38^{-}$) for stem cell transplantation, but the low number of hematopoetic stem cells (HSC) is the most important limitation for its use.[1] Therefore, the use of UCB for allogenic transplantation in adults has been hindered by the concern that a single CB may not contain sufficient numbers of stem/progenitor cells to reconstitute heavier patients in a timely manner.[2] Ex vivo expansion of stem/progenitor cells may circumvent this problem.[3] Because cryopreservation remains the method of choice for long-term preservation of stem/progenitor cells, UCB cryopreservation has become an important issue in banking and transplantation.[4]

Because it would be costly to expand UCB progenitor cells, it is important to determine the feasibility and reproducibility of progenitor cell expansion after cryopreservation. This study has the practical advantage that an expansion procedure may need to be performed in certain cases eg, transplants of heavier patients.[5] Cytokine-mediated

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ex vivo expansion of progenitor cells might overcome the limitation of a low number of progenitor cells present in a single UCB collection.[6] On the other hand, the expansion of cells from UCB units before freezing does not seem practical for the higher costs, as the vast majority of UCB donations are never used. Ottenbreit et al[7] suggested that one colony-forming cell (CFC) population (preferentially responsive to fibroblast condition medium) was destroyed by the freeze–thaw procedure, while another which preferentially responded to leukocyte conditioning medium survived. Rice et al[8] conclude that prior cryopreservation of ex vivo expanded cord blood cells was not detrimental to engraftment as measured in the NOD–SCID mouse model. Bertolini et al[9] reported that cryopreserved UCB committed as well as multipotential progenitor cells retained their potential for ex vivo expansion. Mugishima et al[10] reported that engraftment depended on succesful cryopreservation of cells without substantial losses in viability.

MATERIALS AND METHODS

Cell Source

UCB (n = 10) obtained from the umbilical vein after vaginal delivery or cesarean section was collected in transfer bags containing 25 mL of CPD-A as anticoagulant.

Processing and Isolation of UCB Mononuclear Cells

The UCB samples were red cell–depleted by hydroxyl ethyl starch sedimentation. Solutions of 0.8% HES (Fresenius) in 0.9% Nacl were added directly to the collection bag in a ratio of one part HES to seven parts blood. Low-density UCB mononuclear cells were obtained by Ficoll sedimentation.

Cryopreservation and Thawing

Without any futher manipulation mononuclear cells (MNC; 5 to 10 × 10^6/mL) were diluted (1:1) in ice-cold cryopreservation medium composed of 64% RPMI (Sigma) with 10% DMSC (Merok), 20% human albumin (Bager), 1% CPD-A, and 5% HES in 2-mL cryovials (Nunc). The cells were cryopreserved using an automated programmed control rate freezer (biological freezer BV-25) and a −80 °C mechanical freezer. Samples were immediately placed into the chamber of the cell freezer, which utilized a reference thermoelement that yielded information about the actual sample temprature conditions. The temperature in the freezing chamber was continuously controlled by the coprocessor inside the freezing chamber. Initially, the sample ambient temperature was lowered to 0 °C for 5 minutes followed by 1 °C-reduction per minute to −30 °C with a 20-minute holding period at this intermediate temperature followed by 2 °C-reduction per minute to −70 °C. At the end of the freezing procedures the cells were stored in a vapor phase of nitrogen. The other part of each sample was frozen using a −80 °C freezer. With this method, DMSO treated cells were placed into unolit and then transferred to a −80 °C freezer.

After 30 days, cells were rapidly thawed by and quick shaken in a 37 °C water bath. Once the sample had thawed but was still cool, the cells were washed twice by centrifugation. The cells then were counted for recovery and viability assessed by trypan blue dye exclusion (Merck). $CD34^{+}38^{-}$ content was established by flowcytometery and colony count in semisolid agar media and expansion potential were determined in serum-free media.

Flowcytometery Analysis

Monoclonal antibodies were used for flow cytometery:phycoerythrin(PE)-labeled antiCD38 and FITC-labeled antiCD34 (Dako). Initially 1 × 10^6 MNC/mL washed in PBS-containing 1% FBS (Gibco), 10λ of CD38-PE, and 10λ of CD34-FITC were added to 100λ of cell suspension followed by an incubation for 20 minutes at 4 °C. After washing the cells were fixed with 100λ 1% formaldehyde in 500λ PBS. As negative controls we used PE and FITC antimouse Ab. Ten thousand events were counted with analysis performed on an EPICS XL-MCL flowcytometer. As isotypic controls PE-IgG1 and FITC-IgG1 were obtained from Dako.

Clonogenic Assay

The input MNC and output obtained after liquid culture of fresh and cryopreserved samples were determined using 35-mm tissue culture plates (Sigma) at concentration 1 × 10^5 cells/mL. The medium contained 0.3% agar, 20%FBS, 1% BSA (Sigma) plus 10^{-4} mol/L 2-mercaptoethanol (Sigma) 10 ng/mL SCF, 10 ng/mL IL-3, 10 ng/mL GM-CSF, 1 IU/mL EPO (Stem Cell Technologies, Vancouver, Canada), After 14 days of culture at 37 °C in a 5% CO_2 fully humidified atmosphere, the colonies defined as aggregates of more than 50 cells, were enumerated.

Short-Term Expansion of Stem/Progenitor Cells in Serum-Free Media

MNC (8 × 10^5) cells were incubated in liquid suspension culture for 7 days in SFEM supplemented with recombinant human IL-3 (50 ng/mL), SCF (100 ng/mL), TPO (50 ng/mL), (Stem Cell Technologies) for incubation at 37 °C in an humidified atmosphere of 5% CO_2 without medium exchange or cytokine readdition. Cells were harvested after 7 days, counted and assayed for colony forming cells (CFC), long-term culture-intiating cells (LTC-IC), and expression of CD34+38−. The CD34+CD38− absolute numbers were measured.

Long-Term Culture Initiating Cell (LTC-IC)

Input UCB mononuclear and expanded cells (output) from fresh and cryopreserved samples were evaluated for LTC-IC. Briefly, test cells (2 × 10^4 MNC/mL) were seeded into cultures containing a feeder layer of human bone marrow cells. inactivated with 10 μg/mL mytomycin (Imery). Test cells were resuspended in complete medium consist of IMDM (Sigma) supplemented with 12.5% FBS (Gibco), 12.5% HS (Sigma), 2 mmol L-glutamin (Sigma), 10^{-4} mol 2-ME (Sigma), 0.2 mmol inositol (Merck), 20 μmol folic acid (Merck), and freshly dissolved 10^{-6} mol hydrocortisone (Merck). At a 7-day interval, the cultures were fed by removing half the culture volume followed by replacement with fresh medium. After 4 to 5 weeks in culture, nonadherent and adherent cells harvested by trypsinization were pooled, washed, and assayed for clonogenic elements using semisolid agar cultures.[5] The total number of clonogenic cells present in 4- to 5-week-old-LTC provided a relative measure of the number of LTC-IC that were originally present in the test suspension. Absolute LTC-IC values were calculated by a colony assay.

Statistics

The results was compared with an independent t-test in cryopreserved versus precryopreserved sample. ANOVA or post hoc tests were used for two- or three-group comparisons, respectively.

RESULTS

Flowcytometery Analysis

MNC specimens were analyzed by flow cytometry before and after cryopreservation to determine whether the $CD34^+$ $CD38^-$ cell population was by the freezing and thawing procedures. The dead cells were gated out by considering forward scatter decrement, which had been already evaluated for viability by the trypan blue exclusion dye method. Although the viability (trypan blue exclusion dye) was decreased after cryopreservation, there was no significant difference in the $CD34^+$ and $CD34^+38^-$ absolute numbers as well as the colonogenic capacity and in vitro expansion potential of umbilical cord blood progenitor cells before and after cryopreservation.

Progenitor Colony-Forming Assay

The clonogenic capacity was evaluated before cryopreservation and after thawing by colony culture assays that show differentiation capacity and lineage commitment. Cells were stimulated with a combination of IL-3 (10 ng/mL), GM-CSF (10 ng/mL), SCF (10 ng/mL), EPO (1 IU/mL) in semisolid agar media. The colonies was evaluated morphologically. Each colony was plucked from the plate. After cytospin centrifugation, ≥50 cells were stained by Wright-Giemsa reagent to assess the state of maturation and confirm the existence of a myeloid or an erythroid colony. Benzidine dye was used to identify an erythroid colony. Each plate was covered with benzidine dye. Colonies that became dark after some minutes were identified as erythroid colonies. The samples ($n = 10$) showed no statistically significant difference in the clonogenic capacity of cryopreserved versus precryopreserved samples.

Liquid Suspension Culture

Liquid culture of 8×10^5 MNC cells/mL ($n = 10$) was established in 96-well flat-bottomed plates in the presence of SCF (100 ng/ml), IL-3 (50 ng/mL), and TPO (50 ng/mL). After 7 days, the expansion potential was evaluated by flow cytometery and a colony forming assay. We found 2.04-, 6.3-, 4.47-, and 5.6-fold expansion in the number of MNC, CFC, and CD34+,CD34+38− cells in fresh versus 2.10, 6.64, 4.23, 5.26 fold expansion in cryopreserved samples. After cryopreservation at a nonprogrammed rate in a −80 °C freezer, these items were 1.8, 6.51, 4.7, and 5.68, respectively.

There was no significant difference in CFC fold-expansion between fresh and cryopreserved MNC cells. Similar results were obtained for $CD34^+$ and $CD34^+38^-$ cell expansion from fresh versus cryopreserved MNC cells.

LTC-IC

The frequency of cells that sustained long-term engraftment was measured by LTC-IC assays in the initial MNC UCB cells from fresh versus cryopreserved samples ($n = 3$). The input and output values were 4.67 ± 0.88 and 11 ± 1.73, respectively. After 7 days of liquid culture, the LTC-IC content evaluated by the number of CFC-generated after 4 to 5 weeks in a BM stromal assay was 2.35-fold greater than the input value. In fact, these test confirmed that the expansion procedure succeed in fresh and cryopreserved samples.

DISCUSSION

UCB is a particularly important source of stem cells for ethnic groups that are poorly represented in current bone marrow transplantation registries. One issue in UCB banking is that there is only one opportunity to obtain the sample. Overall, it is limited in volume and cell number. Therefore, efficient transplantation may be limited to children or low weight adults, since the therapeutic dose of stem cells to obtain rapid engraftment in adults may not be reached in cord blood samples. The ability to expand this population of stem cells to reach a therapeutic dose even for adults would be a major advantage that could potentially augment the use of UCB transplantation.

The tolerance of cells to freezing at slow cooling rates depends on their ability to withstand osmotic stress. Granulocytes show much less osmotic tolerance.[11] The survival of $CD34^+$ cells was higher than that of nucleated cells, indicating that $CD34^+$ cells with 10% DMSO are more tolerant to cryopreservation than the total nucleated cell population.[12] Moreover, postthawing it dramatically decreased for the percentage of granulocytes, and highly increased for the percentages of lymphocytes and monocytes. We conclude that the use of DMSO and 10% dextran is harmful to mature cells and cells with large size such as granulocytes, but suitable for lymphocytes and monocytes, especially for cells of small size such as $CD34^+$ cells.[13]

Environmental and chemical changes that occur during cooling alter the membrane's selective permeability. Increased osmotic pressure leads to shrinkage, which increases surface pressure on the membrane. This change could result in alterations including phase separations, collapse, or loss of membrane components, which may or may not be reversible.[14]

To improve the use and reproducibility of $CD34^+$ cell culture procedures at scales sufficient for therapeutic application, several culture parameters were optimized. To eliminate the requirements for fetal calf serum, cell expansion was performed in an serum-free medium (SFEM) which together with exogenous recombinant cytokines offers advantages for ex vivo expansion of cells for clinical purposes by standardizing culture conditions, thereby enhancing reproducibility and removing the risk of contamination for reinfusion into patients.[15]

We evaluated the impact of various growth factors on $CD34^+$ cell cultures. Recombinant growth factors were capable of stimulating progenitor cell expansion and differentiation along various pathways. Stem cell factor interacted with a number of human growth factors to stimulate colony growths. It was particularly effective to stimulate the

formation of mixed cell colonies from $CD34^+$ cells.[16] Moreover, adding other growth factors, particularly human SCF, to the cultures increased expansion of UCB stem cells, decreasing the time needed to generate a sufficient number of stem cells for rapid engraftment of adults.[17]

The Mpl ligands are a family of HGFs that bind to the thrombopoietin receptor, c-*Mpl,* which is related to phenotype of stem cells with long-term repopulating capacity. Tpo was initially defined as a megakaryocyte colony stimulator, but in combination with SCF it enhanced the proliferation of early hematopoietic cells.[12] Also in combination with early acting cytokines, IL-3 improves the ex vivo expansion of UCB stem and progenitor cells.[18]

Finally we observed that MNC, clonogenic cells, $CD34^+$, and $CD34^+38^-$ are enhanced (up to 2.04-, 6.3-, 4.47-, and 5.6-fold versus 2.10-, 6.64-, 4.23-, and 5.26-fold) in fresh versus 2.10, 6.64, 4.23, and 5.26-fold in cryopreserved samples using commercial SFEM in combination with IL-3, SCF, TPO (50, 100, 50 ng/mL). After cryopreservation in a −80 °C freezer, these values were 1.8, 6.51, 4.7, and 5.68. With the use of SCF growth factor, the faster progenitor cell expansion in SFEM could decrease the total volume of expansion medium needed to produce a UCB graft with sufficient HSC for adult transplantation, thereby decreasing the cost of the procedures.[19] Moreover, we concluded that cryopreservation was a good method for cord blood cell storage.[19]

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REFERENCES

1. Yamaguchi M, Hirayama F, Murahashi H, et al: Ex vivo expansion of human UC blood primitive hematopoietic progenitors and transplantable stem cells using human primary BM stromal cells and human AB serum. Cytotherapy 4:109, 2002

2. Preclinical ex vivo expansion of cord blood hematopoietic stem and progenitor cells: duration of culture; the media, serum supplements, and growth factors used; and engraftment in NOD/SCID mice. Transfusion 41:1567, 2001

3. Kim SK, Koh SK, Song SU, et al: Ex vivo expansion and clonality of $CD34^+$ selected cells from bone marrow and cord blood in a serum-free media. Mol Cells 14:367, 2002

4. Wang SY, Hsu ML, Huang MZ, et al: The activity in ex vivo expansion of cord blood myeloid progenitor cells before and after cryopreservation. Acta Haematol 105:38, 2001

5. Aroviita P, Teramo K, Westman P, et al: Associations among nucleated cell, CD34+ cell and colony-forming cell contents in cord blood units obtained through a standardized banking process. Vox Sang 84:219, 2003

6. Denning-Kendall P, Singha S, Bradley B, et al: Cytokine expansion culture of cord blood $CD34^+$ cells induces marked and sustained changes in adhesion receptor and CXCR4 expressions. Stem Cells 21:61, 2003

7. Ottenbreit MJ, Inoue S, Fracassa M: Survival of human colong-forming cells (CFC) after freezing. Cryobiology 16:417, 1979

8. Rice AM, Wood JA, Milrosi CG, et al: Collins. prior crypreservation of ex vivo expanded cord blood cells is not detrimental to engraftment as measured in the NOD-SCID mouse model. J Hematother 10:157, 2001

9. Bertolini F, Lazzari L, Lauri E, et al: Cord blood—derived heamtopoietic progenitor cells retain their potential for ex vivo expansion after cryopreservation. Bone marrow transplantation 15:159, 1995

10. Mugishima H, Harada K, Chin M, et al: Effects of long-term cryopreservation on hematopoietic progenitor cells in umbilical cord blood. Bone Marrow Transplant 23:395, 1999

11. Law P, Alsop P, Dooleg DC, et al: Studies of cell separation. A Comparison of the osmotic response of human lymphocytes and granulocytes monocyte progenitor cells. Cryobiology 20:644, 1983

12. Yang H, Acker JP, Hannon J, et al: Damage and protection of umbilical cord blood cells during cryopreservation. Cytotherapy 3:377, 2001

13. Liu J, Zhou SY, Huang GD, et al: Cryobiological characteristics of cord blood preserved in bioarchive autopreseved liquid nitrogen system. Zhongguo Shigan Xue Ye Za Zhi 1093:261, 2002

14. Introduction to cryobiology. Freezing effect on cellular system. Science Available: www.cem.msu.edu

15. Ho AD, Haas R, Champlin RE: Hematopoeitic stem cell transplantation. New York: Marcel Dekker; 2000, p 300

16. Migliaccio G, Migliaccio AR, Druzin ML: Long term generation of colony forming cells in liquid culture of CD34+ C B cells in the presence of recombinant human stem cell factor. Blood 79:2620, 1992

17. Gilmore GL, Depasquate DK, Lister J: Ex vivo expansion of human U.C.B. and P.B CD34+ hematopoietic stem cells. Exp hematol 28:1297, 2000

18. Provan D, Gribben J: Molecular haematology. New York: Blackwell Science; 2000, p 198

19. Robmanith T, Schroder B, Bug G, et al: Interleukin 3 improves the ex vivo expansion of primitive human cord blood progenitor cells and maintants the engraftment potential of SCID repopulation cells. Stem Cells 19:313, 2001