

Cryopreserved Fetal Human Liver Hematopoietic Progenitor Cells for Repopulating Immune-Deficient Mouse Bone Marrow

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Abstract

Background: Experimental and clinical protocols are being developed for the cryopreservation of human hematopoietic progenitor cells. However, the effect of these procedures on the potential for HPC to repopulate bone marrow is unknown.

Objectives: To examine the effect of cryopreservation on the ability of fetal human liver HPC, which include CD34+ cells and long-term culture-initiating cells, to repopulate immunodeficient non-obese diabetic/severe combined immunodeficiency mouse bone marrow.

Methods: Groups of sublethally irradiated NOD/SCID mice were injected intravenously with cryopreserved or freshly isolated fetal human liver HPC.

Results: Seven weeks after transplantation, flow cytometric analysis of bone marrow samples showed that mice that received the transplanted cells (either cryopreserved or freshly isolated) demonstrated both lymphoid and myeloid differentiation as well as the retention of a significant fraction of CD34+ cells.

Conclusions: Cryopreserved fetal human liver-derived HPC appear to be capable of initiating human cell engraftment in NOD/SCID mouse bone marrow and open the possibility of using cryopreserved fetal human liver HPC for gene manipulation, gene transfusion therapy, and transplantation purposes.

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Hematopoietic progenitor cells are capable of self-renewal, of differentiation into many different cell lineages, and have the potential to treat malignant and non-malignant diseases [1,2]. Fetal human liver HPC are considered a good candidate for genetic manipulation and transplantation [1-5] for these purposes.

The widespread use of various treatment protocols utilizing HPC has resulted in an increased demand for cryobiologic techniques for these cells [6]. However, cryopreservation of HPC with the subsequent analysis of their functional capability has been limited to *in vitro* studies using colony assays or cell cycle analysis studies. Using an *in vivo* model in which sublethally irradiated non-obese diabetic/severe combined immune-deficient mice were injected with HPC resulted in human hematopoiesis. With this model, we can analyze the repopulating characteristics and predict cell performance after transplantation into patients [7-10]. In this study, we describe cryopreserved fetal human liver CD34+ cells and engraftment of long-term culture-initiating cells in NOD/SCID mouse bone marrow.

HPC = hematopoietic progenitor cells
 NOD = non-obese diabetic
 SCID = severe combined immunodeficiency

Materials and Methods

Fetal human liver tissues, CD34+ cell preparation and cell phenotype analysis

Fetal human liver CD34+ cell preparation and phenotype analysis were described previously [6]. Briefly, tissues used in this study were from 16-24 week human fetuses. CD34+ cells were isolated using the MiniMACS CD34 progenitor cell isolation kit (Miltenyi BioTec, Auburn, CA, USA). Column-purified CD34+ cells were analyzed before and after cryopreservation with fluorescein isothiocyanate-conjugated anti-CD34 monoclonal antibody and phycoerythrin-conjugated anti-CD3 monoclonal antibody (Beckman Culture, Brea, CA).

Cryopreservation and thawing of HPC

Cells were frozen in cryopreservation medium containing 7.5% DMSO, 70% fetal bovine serum in Iscove's modified Dulbecco's medium at the cell concentration of 1×10^6 /ml cells [6]. When thawing cells, freezing vials were immediately placed in a water bath at 37°C after removal from liquid nitrogen. An aliquot of cell suspension was diluted 1:1 with trypan blue. The cells excluding the dye were counted. Cells were then washed with the same medium for transplantation.

Transplantation and engraftment

NOD/SCID mice were bred and maintained in the animal facility of Wayne State University School of Medicine (Detroit, MI). Adult mice were sublethally irradiated with 350 cGy from a ¹³⁷Cs source the day before being injected intravenously with human cells. Three mice per group were transplanted with 2.5×10^5 cells/mouse with: a) cryopreserved CD34+ cells, b) freshly isolated CD34+ cells, or c) cryopreserved LTC-IC. At 7 weeks after transplantation, the mice were euthanized and the bone marrow from the femurs of each mouse were collected. The presence of human cells in the mouse bone marrow was assessed by flow cytometry on a FACScan analyzer (Becton Dickinson, San Jose, CA) using human-specific monoclonal antibodies. These include anti-CD45 (FITC) in combination with CD34 (PE), anti-CD3 (FITC)/CD19 (PE), (Beckman Culture, Brea, CA). FITC and PE-conjugated mouse immunoglobulin monoclonal isotypes were used as negative controls.

LTC-IC = long-term culture-initiating cells
 FITC = fluorescein isothiocyanate
 PE = phycoerythrin

Colony-forming assays

All the cell preparations, before and after transplantation, were tested in colony-forming assays that were performed in methylcellulose medium with recombinant cytokines (H4435, Stem Cell Technologies Inc.) as previously described [6].

Stromal feeders and long-term culture assay

A stromal feeder layer comprised of M2-10B4 and SL/SL cells (generously given by Dr. Hogge, D.F., Vancouver, British Columbia, Canada) was used in this study. LTC-IC assays were performed as described [11] after a slight modification. Briefly, 2×10^6 cells per 35 mm were suspended in 2.5 ml of human long-term culture medium with 10^{-6} M freshly dissolved hydrocortisone and then co-cultured on the pre-irradiated confluent feeder cells. The cultures were fed with half-medium changes each week. Total non-adherent and adherent cells were harvested and pooled.

Results

Engraftment of human hematopoietic cells in bone marrow of NOD-SCID mice

Successful engraftment was obtained in all fetal human liver HPC-injected recipients. Figure 1 shows the results of flow cytometric analyses of bone marrow cell profiles in recipients transplanted with 2.5×10^5 cryopreserved fetal human liver CD34+ cells, freshly column-purified fetal human liver CD34+ cells, or fetal human liver LTC-IC. These samples were probed for the presence of human CD45, CD34, CD19 and CD3. Each panel is from one representative animal. Table 1 shows the percentage of engrafted human cells in NOD/SCID mouse bone marrow from the three cell preparations (cryopreserved, freshly column purified CD34+ cells, and LTC-IC). The percentages represent the mean \pm SEM from the three animals in each group. The mean percentages of CD45+ cells were 53.27 ± 4.62 and 50.2 ± 7.04 for both cryopreserved and freshly isolated CD34+ cells, while the percentage for LTC-IC cells was 29.41 ± 6.02 . With respect to the other markers measured, a significant percentage of cells expressed the pre-B cell marker CD19.

Colony-forming activity of CD34+ cells, LTC-IC, and engrafted bone marrow cells

Multi-lineage colonies were observed, after 14 days *in vitro*, from all cell preparations. Mouse bone marrow cells were also checked for colony-forming ability since the human CD34+ phenotype was consistently observed by flow cytometry in all post-transplant preparations. Burst-forming unit-erythroid, G-forming unit-granu-

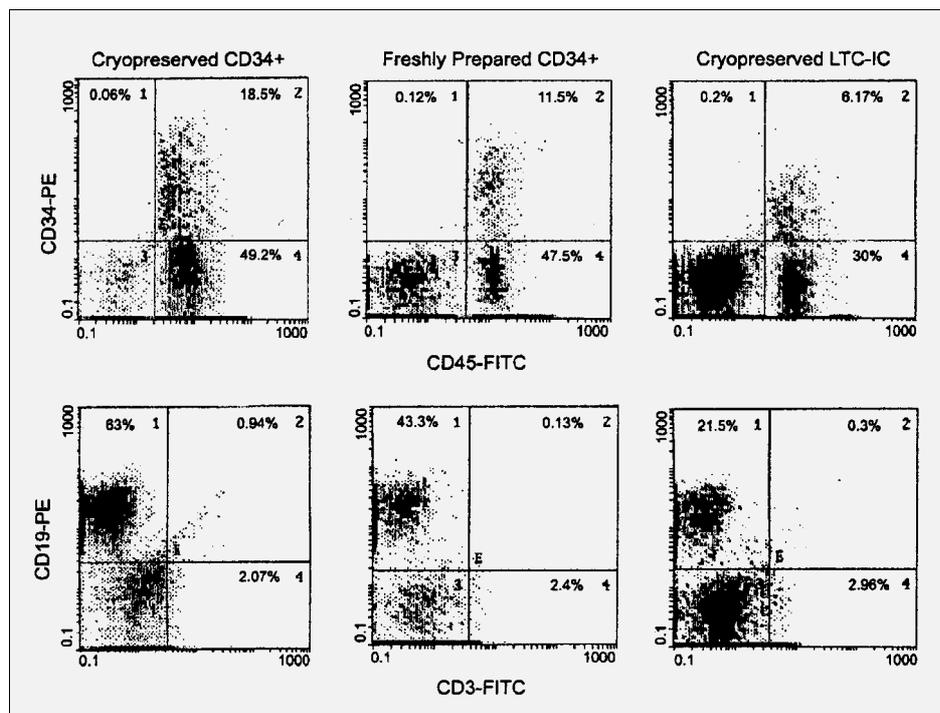


Figure 1. Flow cytometry histogram showing the analysis of cell populations derived from NOD/SCID mouse bone marrow after transplantation with cryopreserved, freshly isolated, or LTC-IC. Individual grafts are from a representative experiment in which mice were transplanted with 2.5×10^5 cells and analyzed after 7 weeks. **First row:** Distribution of CD45 and CD34 cells from mice receiving each cell preparation. **Second row:** Distribution of B cell marker CD19 and T cell marker CD3 from cryopreserved fetal human liver CD34+ cells, freshly column-purified fetal human liver CD34+ cells, and cryopreserved fetal human liver LTC-IC.

Table 1. Overall average percentage of engrafted human cells from NOD/SCID mouse bone marrow

| | CD45 | CD34 | CD19 | CD3 |
|--------|------------------|------------------|------------------|-----------------|
| Medium | 0.63 ± 0.06 | 0.2 ± 70.02 | 0.93 ± 0.48 | 0.63 ± 0.25 |
| Cryo | 53.27 ± 4.65 | 10.13 ± 0.78 | 35.61 ± 2.62 | 2.74 ± 0.11 |
| Fre | 50.2 ± 7.04 | 3.99 ± 0.72 | 13.73 ± 2.24 | 3.69 ± 0.64 |
| LTC-IC | 29.41 ± 6.02 | 8.51 ± 1.59 | 28.09 ± 7.71 | 4.38 ± 1.46 |

Medium = NOD/SCID mice were injected with medium. Cryo = injected with cryopreserved fetal human liver CD34+ cells. Fre = freshly column-purified fetal human liver CD34+ cells. LTC-IC = fetal human liver LTC-IC. Comparing the reconstituted CD45+ cells shows no statistical significant differences between cryopreserved CD34+ cells and freshly purified CD34+ cell transplanted into NOD/SCID mouse bone marrow ($P = 0.12$).

locyte macrophage, and mixed colonies developed from all transplanted mouse bone marrow samples. However, quantitatively, fewer colonies were observed from medium-transplanted mouse bone marrow cells.

Discussion

To determine whether freshly prepared or cryopreserved fetal human liver HPC may have a different capacity for *in vivo* engraftment, we studied the ability of cryopreserved fetal human liver column-purified CD34+ cells and LTC-IC to populate NOD/SCID mouse bone marrow. Some investigators [8] have reported that different microenvironments in NOD/SCID mouse organs or

tissues can give rise to distinct engraftment results. Bone marrow of NOD/SCID mice, however, is the preferred site for human hematopoiesis in this animal model because it contains the highest number of autologous CD34+ cells as well as the highest number of *in vitro* clonable myeloerythroid progenitors [9].

CD45+, as a human leukocyte marker, was detected on bone marrow cells from all recipient mice. Of the various CD45+ subpopulations, cells expressing the CD19+ phenotype, which has been associated with pre- or B cells, was the most prominent marker observed. This confirms other reports showing that B cell maturation takes place in bone marrow, while a large number of immature B cells reside in the spleen [8,9]. In contrast, CD3+ cells were marginally detectable and their percentage was at the level of sensitivity of the flow cytometric assay. These findings suggest that human T cell progenitors are unable to proliferate and differentiate in NOD/SCID mice [12]. We also observed a significant percentage of human CD34+ cells in engrafted mouse bone marrow, though other investigators have proposed that engrafted human cells lack the ability to express CD34+ [10]. In addition, the present results demonstrate that reconstituted CD34+ cells from NOD/SCID mouse bone marrow can produce multi-lineage colonies *in vitro*, and therefore HPC can differentiate into various hematopoietic progenitors including myelocytic, erythroid, megakaryocytic, and multipotential progenitors in NOD/SCID mice [13].

In summary, engrafted NOD/SCID mice that received either transplanted cryopreserved or freshly prepared fetal human liver CD34+ cells demonstrated both lymphoid and myeloid differentiation as well as the presence of CD34+ cells in their bone marrow. Cryopreserved fetal human liver LTC-IC were also capable of repopulating mouse bone marrow. We conclude that cryopreserved fetal human liver CD34+ cells or LTC-IC derived from fetal human liver may provide a source of transplantable cells that are suitable for clinical applications.

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References

- Gilles JM, Divon MY, Bentolila E, et al. Immunophenotypic characterization of human fetal liver hematopoietic stem cells during the midtrimester of gestation. *Am J Obstet Gynecol* 1997;177:619-25.

- Weeks SF, Van Bockstaele DR, Plum J, et al. CD34++ CD38- and CD34+ CD38+ human hematopoietic progenitors from fetal liver, cord blood, and adult bone marrow respond differently to hematopoietic cytokines depending on the ontogenic source. *Exp Hematol* 1998;26:1034-42.
- Huang S, Law P, Young D, et al. Candidate hematopoietic stem cells from fetal tissues, umbilical cord blood vs. adult bone marrow and mobilized peripheral blood. *Exp Hematol* 1998; 26:1162-71.
- Golfier F, Barcena A, Cruz J, et al. Mid-trimester fetal livers are a rich source of CD34+/++ cells for transplantation. *Bone Marrow Transplant* 1999;24:451-61.
- Muench MO, Cupp J, Polakoff J, et al. Expression of CD33, CD38, and HLA-DR on CD34+ human fetal liver progenitors with a high proliferative potential. *Blood* 1997;89:1364-75.
- Zhao J, Hao H-N, Thomas LR, et al. An efficient method for the cryopreservation of fetal human liver hematopoietic progenitor cells. *Stem Cells* 2001;19:212-18.
- Greiner DL, Hesselton RA, Shultz ID. SCID mouse models of human stem cell engraftment. *Stem Cells* 1998;16:166-77.
- van der Loo JC, Hanenberg H, Cooper RJ, et al. Nonobese diabetic/severe combined immunodeficiency (NOD/SCID) mouse as a model system to study the engraftment and mobilization of human peripheral blood stem cells. *Blood* 1998;92:2556-70.
- Ueda T, Yoshino H, Kobayashi K, et al. Hematopoietic repopulating ability of cord blood CD34+ cells in NOD/Shi-scid Mice. *Stem Cells* 2000;18:204-13.
- Goodell MA, Rosenzweig M, Kim H, et al. Dye efflux studies suggest that hematopoietic stem cells expressing low or undetectable levels of CD34 antigen exist in multiple species. *Nature Med* 1997;3(12):1337-45.
- Hogge DE, Lansdorp PM, Reid D, et al. Enhanced detection, maintenance, and differentiation of primitive human hematopoietic cells in cultures containing murine fibroblasts engineered to produce human steel factor, interleukin-3, and granulocyte colony-stimulating factor. *Blood* 1996;88:3765-73.
- Sutherland H, Eaves C, Eaves A. Characterization and partial purification of human marrow cells capable of initiating long-term hematopoiesis *in vitro*. *Blood* 1989;74:1563-9.
- Robin C, Pflumio F, Vainchenker W, et al. Identification of lymphomyeloid primitive progenitor cells in fresh human cord blood and in the marrow of nonobese diabetic-severe combined immunodeficient (NOD-SCID) mice transplanted with human CD34+ cord blood cells. *J Exp Med* 1999;189:1601-10.

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Capsule

Avoiding acetaminophen toxicity

Acetaminophen is a widely used drug for treating pain, inflammation, and fever. However, because it is metabolized into a toxic compound, high doses can cause liver damage. Zhang and co-workers show that this toxicity is enhanced when a nuclear receptor called CAR has been activated, which in turn increases the expression of those enzymes that metabolize acetaminophen.

However, subsequent treatment of mice exposed to high doses of acetaminophen with a CAR inverse agonist blocked toxicity. The study suggests that CAR inverse agonists could be clinically useful to treat acetaminophen-related hepatotoxicity.

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