Evaluation of Hematological Reconstitution Potential of Autologous Peripheral Blood Progenitor Cells Cryopreserved by a Simple Controlled-Rate Freezing Method


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A novel and simple procedure for the controlled-rate cryopreservation of peripheral blood progenitor cells (PBPCs) was introduced. A freezing bag housed in a protective aluminum canister was placed on top of a styrene foam box in the –85°C electric freezer. A second set of samples was kept in cryotubes placed in a double styrene foam box in the same electric freezer. Measurement of the freezing rate in the PB bags and cryotubes demonstrated that this simple method for PBPC cryopreservation provided optimal conditions for both large-scale and small-scale cryopreservation. Within several days after autologous peripheral blood stem cell transplantation, we thawed the cells in the small sample tubes and evaluated the cell viability, the cell recovery, and the recovery rates of hematopoietic progenitor cells (HPCs), such as CD34+ cells and colony-forming unit-granulocyte/macrophage (CFU-GM) colonies. The median duration of cryopreservation was 59 days (range, 14 - 365 days). According to our analysis, infusions of more than 2 × 10^6 CD34+ cells/kg body weight and 0.5 × 10^6 CFU-GM colonies/kg body weight after thawing had favorable influences on the neutrophil engraftment. We have therefore established a simple freezing method for cryopreservation of human PBPCs, which ensures the transplantability of hematopoietic progenitors even after thawing. In vitro HPC assay after thawing is important to evaluate the quality of cryopreservation procedures.

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The rate-controlled programmed freezer is recognized as the standard method for freezing and cryopreserving peripheral blood progenitor cells (PBPCs) (Rowley et al. 1994; Weaver et al. 1995). However, the rate-controlled freezing procedure is both expensive and time-consuming. As an alternative, several groups have collaborated in the development of a simple method for the cryopreservation of PBPCs in a –85°C mechanical freezer for autologous peripheral blood stem cell transplantation (PBSCT). Some studies on simple cryopreservation methods have shown reproducible hematopoietic recoveries after PBSCT (Makino et al. 1991; Takaue et al. 1994; Cilloni et al. 1999; Choi et al. 2001; Montanari et al. 2003). Our group recently provided a freezing rate of approximately 1°C/min for cord blood (CB) cryopreservation by storing the freezing bag in a protective aluminum canister wrapped with heat-insulating materials and placing sample tubes in a double styrene foam box inside a –85°C electric freezer (Itoh et al. 2003). For the past few years, the same procedure has been introduced into PBPC cryopreservation. In the present study we tested the effects of this simple freezing method by thawing the small samples within several days after autologous PBSCT and evaluating the cell viability, the cell recovery, and the recovery rate of hematopoietic progenitor cells (HPCs), such as CD34+ cells and colony-forming unit-granulocyte/macrophage (CFU-GM) colonies. To extend our study, we also analyzed the capacity of the thawed PBPCs in reconstituting hematopoiesis following myeloablative chemotherapy, because many of the reported cases of hematopoietic reconstitution have been estimated based on the number of CD34+ cells collected before freezing.

Patients and Methods

Patients and apheresis

The retrospective analysis was based on the data requested by the PBSCT study approved by the Institutional Ethics Committee. From May 2001 until April 2003, a total of 31 leukaphereses were performed in 19 consecutive patients in the authors’ division. Informed consent was obtained from each patient or his/her parents before apheresis. There were 14 males and 5 females with a median age of 11 years (range: 1-57). Underlying diseases were: neuroblastoma (n = 3), malignant lymphoma (n = 6), testicular tumor (n = 1), rhabdomyosarcoma (n = 1), Wilms’ tumor (n = 1), Ewing sarcoma (n = 1), malignant rhabdoid tumor (n = 1), hepatoblastoma (n = 1), medulloblastoma (n = 1), Askin tumor (n = 1), extragonadal germ cell tumor (n = 1), and retinoblastoma (n = 1).

PBPCs were mobilized with chemotherapy followed by recombinant human granulocyte-colony-stimulating factor (G-CSF). All patients received filgrastim at a dose of 400 μg/m² body surface area or lenograstim at a dose of 10 μg/kg body weight until the completion of the apheresis procedures. Leukapheresis was performed with a continuous-flow blood cell separator (Spectra AutoPBSC™, GAMMRO BCT, Englewood, CO, USA) for all the procedures (Kudo et al. 2003). The Spectra AutoPBSC™ System provides a disposable set with low product volumes and a low extracorporeal volume, thereby reducing the need to perform a blood prime. The instrument was used to calculate the patient’s total blood volume (TBV) according to weight, height, and sex, and the completion of the procedure was set at 2-fold the patient’s TBV, calculated after deducting the ACD-A volume. Leukapheresis was continued daily in order to collect the target number of CD34+ cells within three successive days.

Cryopreservation and thawing of PBPCs

RPMI 1640 medium (GIBCO™, Grand Island, NY, USA) and Heparin 2,000 U were added to the PBPC collection bag to a total volume of 100 mL. Apheresis components were kept cold with wet ice and gently mixed with an equal volume of cryoprotective solution consisting of 12% hydroxyethyl starch (HES), 10% dimethyl sulfoxide (DMSO), and 8% human serum albumin (HSA) (Wellfide, Osaka) over the course of 15 min using a syringe pump and an orbital mixer to assure smooth but vigorous mixing. The bag contained a volume of 200 mL to give final concentrations of 6% HES, 5% DMSO, and 4% HSA with nucleated cell concentrations ranging from 4.75 to 220 × 10^6/mL. After the mixing, the cell suspension was transferred to a series of freezing bags (F-100, Nipro, Co., Osaka) in 100 mL volumes. A protective aluminum canister housing the freezing bag was deposited horizontally on a level surface on the top of a styrene foam box inside a –85°C electric freezer (Sanyo Electric Co., Tokyo). At the same time, two aliquots of 1.0 mL stored in polypropylene vials (CryoTube; Nalge
Nunc, Rochester, NY, USA) were placed in a double styrene foam box inside the same electric freezer for quality evaluation. After more than 3 hours of cooling the blood cells at below –80°C, the bags and vials were both introduced into a rack in the same mechanical freezer. The freezer had a system of visual and acoustic alarms and a graphic register of temperature. The temperature during cooling was recorded by Thermorecorderwide (RT-50, Tabai ESPEC Co., Osaka) with a thermosensor (RTH-5040, Tabai Espec Co.) inserted into dummy bags and dummy sample tubes (Itoh et al. 2003). The freezing curves were analyzed with Microsoft Excel software (Microsoft Excel, Microsoft Systems, Mountain View, CA, USA).

The cells in the sample tubes were thawed by rapid immersion in a 37°C water bath. Immediately after thawing, 0.5 ml of each concentrate was transferred to a 1.5-ml polypropylene tube (BM Bio, Tokyo), diluted with 0.5 ml of a solution containing 5% (wt/vol) HSA in an isotonic salt solution with continuous mixing, placed on ice for 5 min, and centrifuged at 400 × g for 10 min. Half of the supernatant was removed and the sedimented cells were slowly resuspended in the residual solution. The cell counts were determined with an automated hematology analyzer (T-890, Coulter Corp., Miami, FL, USA). We applied the trypan blue dye exclusion test to differentiate dead and live cells. A minimum of 500 unstained viable cells and stained dead cells were counted with an improved Neubauer hemocytometer (Heinz Herenz Medizinalbedarf, Hamburg, Germany), and cell viability was expressed as a percentage of the total cells counted.

**Enumeration of CD34⁺ cells**

The CD34⁺ cell count in the PBPC products was evaluated by flow cytometry. In brief, the nucleated cells were incubated for 30 min at 4°C with a CD34 monoclonal antibody conjugated with phycoerythrin (PE) (8G12, IgG1, Becton Dickinson Immunocytometry Systems, San Jose, CA, USA) and a CD45 monoclonal antibody conjugated with fluorescein isothiocyanate (FITC) (2D1, IgG1, Becton Dickinson). PE- or FITC-conjugated mouse monoclonal IgG1 (PharMingen, Becton Dickinson Co., San Diego, CA, USA) was used as an isotype-matched negative control. Red blood cells were lysed with Tris ammonium chloride lysing reagent and washed in phosphate-buffered saline. The stained cells were immediately analyzed using a flow cytometer (FACS Calibur, BD Pharmingen, San Diego, CA, USA) with software (Cell Quest, Becton Dickinson). For cytometric settings, side scatter (SSC) was collected in a linear mode and signals generated by the FITC-CD45 were collected in a log mode. CD34 fluorescence was assayed on CD45⁺ cells selected by the fluorescent mode versus the SSC gate. At least 60,000 events were analyzed in each sample.

**Hematopoietic progenitor cell assay**

Assays for CFU-GM, burst-forming unit-erythroid (BFU-E), and colony-forming unit-granulocyte/erythroid/macrophage/megakaryocyte (CFU-GEMM) were performed before and after cryopreservation, as previously described (Katayama et al. 1997). Briefly, PBPC cells were plated in a 35-mm plastic dish (Falcon, Becton Dickinson Labware, Franklin Lakes, NJ, USA) at a concentration of 50 to 60 CD34⁺ cells/ml in 1 ml of a ready-made culture medium (MethoCult GF H4434, Stem Cell Technologies, Vancouver, Canada). The dishes were maintained at 37°C under a humidified atmosphere with 5% CO₂ in air. After 14 days in culture, CFU-GM, BFU-E, and CFU-GEMM colonies of 40 or more cells were enumerated under an inverted microscope and counted separately in the same dish.

Post-thawing recovery rates of CFU-GM were calculated as follows:

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\text{Recovery rate (\%) = } \frac{\text{total number of CFU-GM after thawing}}{\text{total number of CFU-GM before freezing}} \times 100.
\]

**Statistical analysis**

Statistical analysis was performed using software (Microsoft Excel, Microsoft Systems, Mountain View, CA, USA). The Student’s t-tests for unpaired data were used to statistically evaluate the results on cryopreservation. A p value of less than 0.05 was considered significant.

**RESULTS**

**Freezing investigations in the –85°C freezer**

Our preliminary data showed that the direct placement of a protective aluminum canister housing the bag and sample tubes in the –85°C electric freezer provided less than optimal cooling conditions, resulting in a low cell viability and low recovery of CD34⁺ cells and colony-forming cells (data not shown). To define the optimal conditions, the temperature during cooling was moni-
stored in 4 bags in the canisters lying on top of a styrene foam box as dummy samples and 3 sample tubes placed in a double styrene foam box inside the –85°C electric freezer. Fig. 1 shows curves of the freezing temperature representing the mean values for cryopreservation. Potentially acceptable cooling curves were obtained in the freezing bags and sample tubes with mean transition times of 4 min (range, 3-5 min) and 4 min (range, 2-7 min), mean pre-transition freezing rates of 3.4°C/min (range, 1.6-4.8°C/min) and 1.6°C/min (range, 1.3-2.0°C/min), and post-transition freezing rates of 1.3°C/min (range, 1.2-1.5°C/min) and 1.3°C/min (range, 1.1-1.5°C/min), respectively. The simple storage technique yielded a kind of controlled-rate freezing, which was named a simple controlled-rate freezing (Itoh et al. 2003).

Cryopreservation efficacy

The recovery rates of CD34⁺ cells and CFU-GM colonies were assessed for 44 PBPC products transplanted to 19 patients. The median duration of cryopreservation was 59 days (range, 14-365 days). The mean cell viability after thawing was 90.0% (s.d., 6.2%). The median absolute cell counts before freezing and after thawing were $46.50 \times 10^8$ (range, $3.25 \times 10^8$ - $220.0 \times 10^8$) and $41.38 \times 10^8$ (range, $1.00 \times 10^8$ - $185.0 \times 10^8$), respectively. The mean recovery rate was 83.9% (s.d., 16.6%). The mean recovery rates of CD34⁺ cells and CFU-GM colonies was 96.6% and 87.4% (s.d., 32.7% and 29.4%), as shown in Table 1.

| Table 1. Cryopreservation efficacy of CD34⁺ cells and CFU-GM colonies (n = 44) |
|---------------------------------|-----------------|-----------------|-----------------|
|                                 | Before freezing¹ | After thawing¹ | Recovery rate (%)¹ |
| Cell viability (%)              | 98.4 ± 2.8      | 90.0 ± 6.2      | /               |
| CD34⁺ cells (× 10⁶/kg)         | 3.83 ± 3.82     | 3.61 ± 3.62     | 96.6 ± 32.7     |
| CFU-GM colonies (× 10⁶/kg)     | 0.73 ± 0.69     | 0.63 ± 0.65     | 87.4 ± 29.4     |

¹Mean ± s.d.
Engraftment time

Nineteen evaluable patients received 37 autologous PBSC infusions with 44 individual PBPC products after myeloablative chemotherapy. Five patients received two PBPC products at the same time due to low cell dose. G-CSF (filgrastim at a dose of 400 μg/m² body surface area or lenograstim at a dose of 10 μg/kg body weight) was administered after the PBSCT to obtain a neutrophil recovery of > 1 × 10⁹/liter. Hematopoietic recovery was obtained in all of the patients. The mean estimated doses of CD34⁺ cells and CFU-GM colonies were 2.18 × 10⁶/kg body weight and 0.39 × 10⁶/kg body weight, respectively. The median recovery time for a neutrophil count of 0.5 × 10⁹/liter was 13 days (range, 4 - 23 days). The hematological reconstitution potential in transplantation with CD34⁺ cells in doses exceeding 2.0 × 10⁶/kg body weight was comparable to that with CFU-GM in doses exceeding 0.5 × 10⁹/kg body weight; the median recovery time for a neutrophil count of 0.5 × 10⁹/liter was 10.5 days in both transplants. The transplants with CD34⁺ cell counts of less than 2.0 × 10⁶/kg body weight were significantly associated with delayed engraftment (p = 0.0002). The median recovery time for a platelet count of 50 × 10⁹/liter was 15.5 days (range, 6 - 42 days). All but one of the thirteen evaluable patients with platelet engraftment received transplants of CD34⁺ cells at doses exceeding 1.40 × 10⁶/kg body weight. The other patient received CD34⁺ cells at a dose of 0.5 × 10⁶/kg body weight with a platelet engraftment time of 36 days. In six patients with progressive disease after transplantation, the platelet count did not reach 50 × 10⁹/liter by the last follow-up day.

Discussion

Cryopreservation of hematopoietic cells is required for almost all autologous blood cell transplants and for some allogeneic blood cell transplants. Cryopreservation is usually performed according to controlled-rate methods with a rate-controlled programmed freezer. Controlled-rate freezing is followed by storage of hematopoietic cells in nitrogen in either the liquid or vapor phase. The procedures are both expensive and time-consuming. As an alternative, a simple method of cryopreservation using a mechanical freezer has been introduced for the PBSCT procedure (Makino et al. 1991; Takaue et al. 1994; Cilloni et al. 1999; Choi et al. 2001; Montanari et al. 2003). One important disadvantage of uncontrolled-rate freezing is the lack of recordable data on the cooling rate. Our group recently obtained a cooling rate of approximately 1°C/min for CB cryopreservation by wrapping heat-insulating materials around the protective aluminum canister in which the freezing bag was kept and placing sample tubes in a double styrene foam box inside an −85°C electric freezer (Itoh et al. 2003). The cryopreservation of CB small samples by this simple freezing method may even have the potential to enable the transplantation of hematopoietic progenitors after thawing. The purpose of the present study was to find optimal freezing conditions and investigate whether the simple controlled-rate freezing of PBPC cells at −85°C without a rate-controlled programmed freezer could be applied for the cryopreservation of PBPCs for transplantation.

Clark et al. (1991) reported that marrows may be preserved even more effectively in a −70°C freezer by limiting the transition or plateau phase to the shortest possible duration and maintaining a cooling-rate of between 1 and 2°C/min after transition. Makino et al. (1991) reported that an optimal cooling condition may be obtained by adding HES to large-scale samples of peripheral blood mononuclear cells in 100-ml freezing bags when the duration of the post-freezing plateau was kept below 4 min and the post-plateau cooling rate was kept under 3.3°C/min. As reported in the results, we obtained a cooling curve with a transition time of 4 min and cooling rate of 1.3°C/min in a −85°C electric freezer by storing the freezing bags in canisters placed on top of a styrene foam box and storing sample tubes inside a double styrene foam box. Based on this result, we employed this simple storage freezing method for the cryopreservation of PBPC cells. In our analysis of the PBPCs cryopreserved in the bags for clinical purposes after a median storage of 59
days, we found that the marrow function was reconstituted in all of the transplant patients. The simple controlled-rate cryopreservation allowed a high progenitor cell recovery, and no relevant detrimental effect was evident in vitro on the post-thawing recovery of hematopoietic progenitors. These findings suggest that this simple freezing procedure helps protect the hematopoietic progenitor cells against substantial damage. Numerous groups have established that rapid hematopoietic reconstitution following myeloablative chemotherapy depends on the number of CD34+ cells collected (Gordon et al. 1995; Bensinger et al. 1996). A dose of $2.0 \times 10^6$ CD34+ cells/kg body weight at the time of harvest has been identified as the minimum target dose required to ensure rapid engraftment. However, no studies have established the minimum threshold required during reinfusion after thawing. Cryopreservation and processing of autologous stem cell collections significantly reduces the number of viable CD34+ cells available for reinfusion (Allan et al. 2002). While our data fail to identify a lower threshold of CD34+ cells required to ensure successful engraftment, they suggest that fewer than $2.0 \times 10^6$ cells/kg body weight post-thaw may be sufficient. Further studies will need to be conducted to evaluate the association between the number of thawed CD34+ cells and the rates of hematopoietic engraftment following autologous PBSCT. We must note, however, that a significantly delayed neutrophil engraftment was observed in patients with doses of CD34+ cells and CFU-GM colonies lower than $2.0 \times 10^6$ and $0.5 \times 10^6$ /kg body weight after thawing respectively. A quantitative relationship between the number of thawed CD34+ cells and the time to platelet engraftment could not be analyzed due to the small number of evaluable patients in our study.

In summary, the simple freezing method can be used to cryopreserve PBSC for transplantation, and the number of CD34+ cells from cryovials after thawing is evidently associated with the time to neutrophil engraftment in autologous PBSCT. The measurement of hematopoietic progenitors after thawing may be useful for the quality control assessment of stem cell cryopreservation.

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mal conditions for both large-scale and small-scale cryopreservation of umbilical cord blood cells. *Transfusion, 43*, 1303-1308.


