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Pooled umbilical cord blood as a possible universal donor for marrow reconstitution and use in nuclear accidents

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Abstract

Human umbilical cord blood has been shown to be an effective source of stem cells for marrow reconstitution in pediatric patients. Unfortunately, the quantity of stem cells obtained from an individual donor can be quite limited in both the total volume and the numbers of stem cells per ml of cord blood. HLA matching further limits the availability, but recent publications indicate close matching may be unnecessary. Therefore, if cord blood from different donors can be combined, larger numbers of stem cells can be available for clinical use provided pooling does not produce a negative effect. Storage of single cord blood specimens at 4° C for 10–21 days in gas permeable bags produced an apparent increase in the percentage of immature cells (CD34, CD117, GPA) and mitotic activity (S+G2/M cells) over day 1. With similar storage of pooled specimens there was a further increase in the number of immature colonies cultured, CD34, CD117, GPA, S+G2/M cells. In addition, nucleated red blood cells increased over the mean values obtained from single cord blood samples. Our previous studies have indicated that large numbers of human mononuclear cells are necessary to reconstitute an irradiated animal model. By combining multiple samples of human cord blood, adequate numbers of stem cells could be pooled for use in adults and would provide cells for megadose therapy, including those patients that had accidentally received lethal irradiation. © 2001 Elsevier Science Inc. All rights reserved.

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Introduction

The primary barrier to the use of umbilical cord blood, instead of bone marrow, for transplantation is the difficulty in obtaining large enough quantities of stem cells with an appropri-

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ate tissue type (matched HLA) to effect a successful allograft. Clinically it is presumed, the presence of an adequate number of stem cells, closely matched to the recipient, should provide an effective marrow transplant without significant graft vs. host disease (GVHD). This concept applies to both children and adults (1). However, the number of stem cells required are far greater for an adult than a child. Currently, there is no exact identification of the pluripotential stem cell, but they have been estimated to be in much greater numbers in human umbilical cord blood than in adult bone marrow (2). Another impediment for the use of cord blood for marrow transplantation is that it has been noted that individual samples of umbilical cord blood are quite variable in quantity, both as to the volume obtained from a single donor and the numbers of colony forming cells per ml of cord blood (3), thereby limiting the usefulness of a single donor even if appropriately matched.

Review of our data indicated that human cord blood, stored for 5–7 days at 4° C, produced survival of mice that had been lethally irradiated, and that survival was directly related to the dose of mononuclear cells administered (4) (Fig. 1). In addition, after 21 days of storage a 4° C, cord blood could still produce secondary blast and mixed cell colonies (5). Furthermore, storage in gas permeable bags at 4° C also produced a decrease in T cells and a marked decline in cell reactivity to mitogens and alloantigens in mixed lymphocyte cultures after 14 days of storage (6). However, significant animal (mouse) survival could still be obtained with the use of human cord blood even after storage at 4° C for 21 days (5). Therefore, if cord blood cells

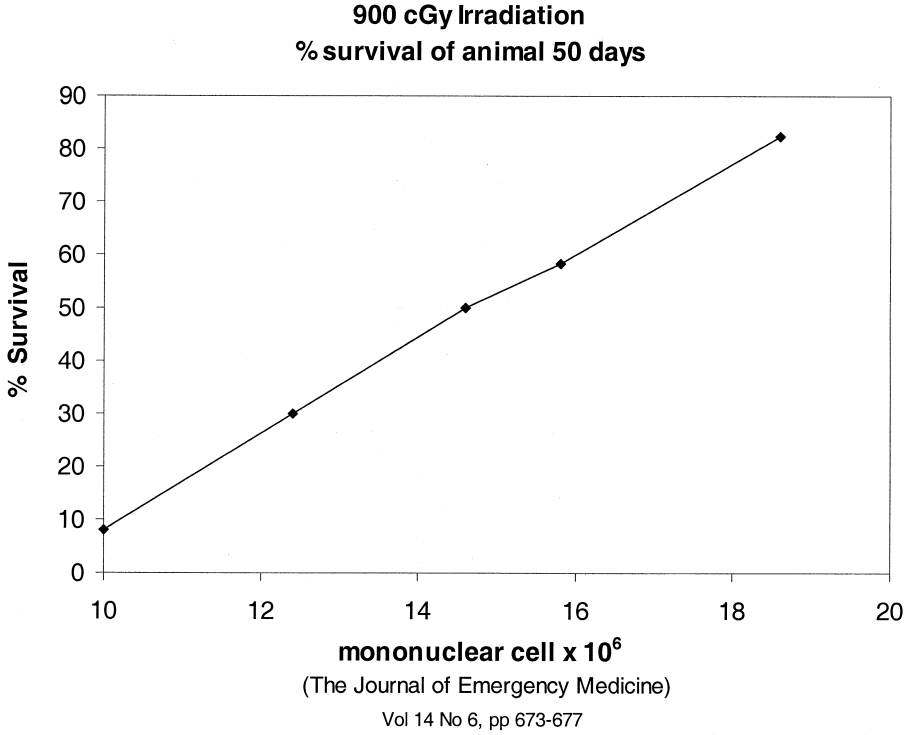

Fig. 1. Survival of mice following lethal irradiation and treatment with human cord blood mononuclear cells.

could be pooled, potentially this would allow the collection of adequate numbers of stem cells for use in both children and adults, and would make it available to all in need of a marrow transplant (7) including those exposed to nuclear accidents. Therefore, it would be important to determine that pooling and storage of human umbilical cord samples, unmatched for HLA, did not produce a negative effect in vitro. This is the purpose of this study.

Materials and methods

To determine if there were any adverse effects from the pooling of cord blood samples, the following experiment was undertaken. Fifteen human umbilical cord blood samples were obtained from the placentas of normal full-term neonates and collected into 50ml sterile polypropylene test tubes containing 5ml citrate phosphate dextrose (CPD) as an anticoagulant. Specimens varying from 30 to 40cc, were received in the laboratory within 48 hours after the birth of the infants and kept at room temperature. Upon receipt, 5ml were removed from each specimen on the day received, mixed in combination with two or three different single specimens and transferred into gas permeable polyolefin blood bags (Cryocyte™ Freezing Container, Baxter Healthcare Corp., Deerfield, IL). By this method, all fifteen single specimens were combined into six units and portions of each single specimen were maintained. There were three bags, each representing a combination of three specimens and three bags representing a combination of two single specimen. Two combinations of specimens were incompatible blood groups and four were compatible blood groups.

Twenty-four hours later (DI) the combined blood samples were each subjected to a cell culture, cell count, smear, and flow cytometry for phenotyping. All combined specimens were stored at 4° C for the same period of time under the same conditions as the individual stored samples. Similar measurements were made on day 10 (D10) and day 21 (D21). Day 21 was chosen arbitrarily as historically, peripheral blood at one time was stored for 21 days.

Cell culture

Cord blood from individual samples (n=15) and combined cord blood (n=6) were all stored at 4° C in Cryocyte Freezing Containers. Clonal growth was determined on day 1 and sequentially on day 10 (D10) and day 21 (D21). Low-density (1.077) cells were isolated through histopaque (Sigma, St. Louis, MO) density-gradient centrifugation at 1500rpm for 30 minutes. The low-density cells were harvested, washed twice with 2% FBS-IMDM, (Fetal Bovine Serum-Iscove's Modified Dulbecco's media) and then plated at 1×10^5 cells/ml in semi-solid culture medium in triplicate. The IMDM culture medium was supplemented with 30% FBS and 1% methyl cellulose. Dishes were incubated at 37°C in 5% CO_2 with full humidity for 14 days. Various cytokines were used individually, or in combination in this culture system as follows. Human recombinant GM-CSF (GM) 100U/ml (Sigma, St. Louis, MO), Human recombinant IL-3 100U/ml (Sigma), human recombinant EPO IU/ml (Procrit, Amgen, Thousand Oaks, CA) and a combination of GM+IL3, EPO+IL3 and GM+EPO+IL3.

Colonies were scored under low power phase inverted microscopy after two weeks of incubation. CFU-GM colonies were defined as aggregates consisting of 50 or more cells. Cell aggegates that had the red color of hemoglobin and contained at least 50 cells, or were comprised of at least three sub-colonies containing a minimum of 10 cells each, were scored as

BFU-E. CFU-GEMM colonies appeared to have both erythroid and non-erythroid cells, but shared a single growth center. A colony consisting of more than 40 cells that appeared colorless, transparent, with a uniform small round shape and with no sign of terminal differentiation were counted as a blast cell colony (CFU-BL).

Flow cytometry

Total white blood cell count (WBC) was determined by cell-Dyn 4,000, Abbot Laboratories, Inc. (Santa Clara, CA) which excludes nucleated red blood cells. Three color flow cytometric analysis for surface markers and for cell cycle analysis using propidium iodide was performed. For surface markers, cord blood (100ul) was stained with 10ul of flourochrome-conjugated monoclonal antibodies. Antibody combinations included CD34-FITC/Glycophorin A-Pe/ CD45-PerCP, CD34-FITC/CD117-PE/CD45-PerCP, HLA-classI-FITC/CD34-PE/CD45-PerCP (Glycophorin and CD117 antibodies from Immunotech, Hialeah, Fl; anti-HLA from Pharmigen, San Diego, CA; CD34 HPCA2 and CD45 antibodies from Becton-Dickinson, San Jose, CA). The mature erythrocytes were lyzed using FAC Lysing solution according to manufacturer's directions (Becton Dickinson) and the rest of the sample fixed with 0.5% paraformaldehyde. After standing for 30 minutes, the cells were analyzed on Becton-Dickinson FACS Calibur flow cytometer. Dot-plots of side scatter vs. CD45 were used to gate only CD45+ cells. Only CD45+ gated cells were analyzed. The data are expressed as % positive gated cells and calculated cell concentration/mm^3. Cell cycle analysis was accomplished using ModFit LT software (Verity, Topsham, ME). Percent S-phase and G2/M were noted to detect replicative ability. Doublets were excluded by gating.

Statistics

The P values are based on 2-tailed student's t-test.

Results

In specimens stored for 21 days at 4° C, despite a decline in the absolute number of colonies, the percentage of primitive colonies CFU-BL (colony forming units-blast cell) remained relatively constant. When the combined cord blood samples were compared to the individual samples; both stored for the same time periods, there was statistical evidence that the combined samples after storage for 21 days at 4° C had an increase in the percentage of colony forming units of CFU-BL and CFU-BL + GEMM (Table 1). Combining the samples not only indicated that there was no decrease in the primitive colonies but, in fact, indicated a significant increase in the number of primitive colonies (Table 1). Somewhat similar data were obtained when cord blood was stored in Teflon bags (American Flouroseal Corp., Gaithersburg, MD), which are gas permeable. The colony formations and response to cytokine of the 15 cases described herein (that had simultaneous flow cytometry studies) were consistent with our previously published studies (5,6).

After storage for 21 days, the percentage of CD34 +, CD117+, GPA+ and evidence of mitotic activity (S+G2M) with single specimens increased, while the percentage of other phenotypes and overall viability markedly decreased. When the cord blood samples were combined, the absolute number of GPA+ and S+G2M+ cells per milliliter of cord blood

Table 1
Hematopoietic colonies from human cord blood, single specimens (n = 15)

CYTOKINES ADDED	D1 GM	D1 BFU-E	D1 GEMM#	D10 GM	D10 BFU-E	D10 GEMM	D10 BL	D21 GM	D21 BFU-E	D21 GEMM	D21 BL
GM	62.4± 16.9	0	0	32.3 ±8.1	0	0	3.6± 2.0	3.2± 1.8	0	0	1.73± 1.1
GM+IL3	127.0± 12.1	0	0	63.5± 9.5	0	0	4.2± 1.4	6.2± 1.2	0	0	4.33± 2.6
EPO	9.8 ±4.3	10.7± 6.8	66.1± 6.4	3.2± 2.1	4.8± 1.8	22.9± 3.9	0	0.7± 0.1	0.9± 0.6	7.5± 2.1	0
EPO+IL3	27.0± 4.6	18.5± 6.8	98.1± 8.1	11.4± 1.8	5.4± 1.2	56.7± 7.8	1.2± 1.0	1.3± 0.5	1.7 ±0.4	24.33± 5.2	1.6± 0.9
G+E+IL3	43.6± 5.3	9.0± 2.5	101.6 ±5.8	18.6± 5.1	8.2± 1.5	72.8± 6.4	3.9± 1.1	2.9 ±1.2	3.0± 1.2	26.9± 6.4	2.6± 0.8

15 individual specimens combined into 6 units

CYTOKINES ADDED	D1 GM	D1 BFU-E	D1 GEMM#	D10 GM	D10 BFU-E	D10 GEMM	D10 BL	D21 GM	D21 BFU-E	D21 GEMM	D21 BL
GM	19.66± 13.8	0	0	36.6± 2.5	0	0	4.0± 1.3	4.1± 2.0	0	0	2.1± 1.5
GM+IL3	83.0± 12.3	0	0	79.5± 6.4	0	0	4.3± 1.8	5.1± 1.3	0	0	4.8± 1.8
EPO	15.66± 3.8	11.6± 2.3	100.8± 5.7	6.3± 2.0	4.8± 3.1	42.8± 5.8*	0	0	1.0± 0.6	7.3± 0.7	0
EPO+IL3	31.0 ±2.5	19.0± 4.6	119.0± 8.5	19.66± 3.8	6.6± 2.1	90.3± 3.8**	4.6± 1.0*	2.1± 2.1*	1.1± 0.5	27.6± 1.3*	2.3± 0.9
G+E+IL3	45.83± 3.2	11.0± 2.6	137.5± 6.3	26.0± 6.6	10.0± 4.6	95.6± 6.7*	4.16± 1.5	2.3± 0.8	3.1± 0.74	34.6± 0.8**	3.0 ±0.8

The combined specimens significantly better than singles *** p<0.05 ** p<0.01**
Colony forming unit: GM(Granulocytic-Monocytic); BFU-E(Brust Forming Unit-Erythoid);
GEMM(Granulocytic/Erythocytic Megakaryocytic/Monocytic); BL(Blast)
#: Blast colonies connot be identified in day 1 colonies.

actually increased, possibly indicating some growth factor or other stimulation when the samples were combined (Table 2). Incompatible blood groups appeared to have no direct relationship with the end results at day 10 and day 21, but would not be recommended for clinical use at this time as this could potentially introduce a long-term sensitivity to the ABO blood groups (9).

Table 2
Cold blood specimen

SINGLE	D1	D10	D21
WBC/mm^3	6,366 ±26.88	1,517± 32.44	790.6 ±12.45
CD34 %	0.91± 1.87	4.49 ±1.56	3.91±0.95
CD34/mm^3	57.9	68.1	30.9
S+G2/M %	1.31± 0.70	2.99 ±1.52	2.69 ±0.62
S+G2/M/mm^3	83.51	45.46	29.79
CD117 %	0.32 ±0.89	1.99 ±0.88	2.87 ±1.15
CD117/mm^3	20.37	30.2	22.69
GPA+ %	0.71 ±1.0	5.86± 4.75	5.90±0.87
GPA+/mm^3	45.19	88.93	46.64
Combined			
WBC/mm^3	3,097± 8.88	2,681±10.55*	955.3± 12.44
CD34 %	1.65± 2.31	3.60± 1.33	5.67± 0.99*
CD34/mm^3	51.1	96.5*	54.16**
S+G2/M %	1.40± 1.20	3.49 ±1.03*	6.31± 0.32**
S+G2/M/mm^3	43.35	52.96	60.27**
CD117 %	0.96 ±0.78	3.18± 0.86	4.95± 0.88
CD117/mm^3	29.73	85.2**	47.28**
GPA+ %	0.71± 0.85	2.58 ±1.34	18.78± 5.34***
GPA+/mm^3	21.98	69.1	179.41***

The combined specimens significantly better than singles
*** p<0.05 ** p<0.01 ***p<0.001**
± : Standard Error
D: Days of storage at 4° C in gas prmeable bags

Although at day 10 of storage at 4°C there was a significant increase of normoblasts in the differential counts at day 21, there still remained a significant increase in normoblasts per W.B.C. This paralleled the percentage of GPA+ cells per mm^3, which also significantly increased. In single cord blood samples, the percentage of nucleated red blood cells (RBC) per milliliter statistically increased (7.9% to 44% $P<0.01$) from day 1 to day 21. In the single specimens stored for 21 days, not only was the percentage increased, but also the absolute number of nucleated RBC increased. In the combined specimens, there was an even greater increase in nucleated red blood cells over the single specimens (628.8/mm^3 compared to 350.2/mm^3 $P<0.05$) (Fig 2).

Discussion

The enormous potential using umbilical cord blood for transplantation has been documented in the past decade (10). However, some of the problems that remain to be addressed before cord blood (CB) transplantation becomes more widely used includes the potential for HLA-mismatched CB cells to cause GVHD. Furthermore, the number of stem cells that can be obtained from a single CB sample for transplantation, the known wide variation in the number of cells per unit of cord blood, and the questionable efficiency of hematopoietic progenitor cells in CB that are cryopreserved for prolonged periods of time are all limiting factors. By pooling specimens and storing at 4° C in a gas permeable bags, these problems may be eliminated.

The findings presented here would suggest that there was mitotic activity and some cellular expansion with human umbilical cord blood after storage at 4° C for 10 to 21 days in gas permeable bags. If human cord blood could be potentially combined it would provide all patients, adults or children of different ethnic origins, with a sufficient number of stem cells for

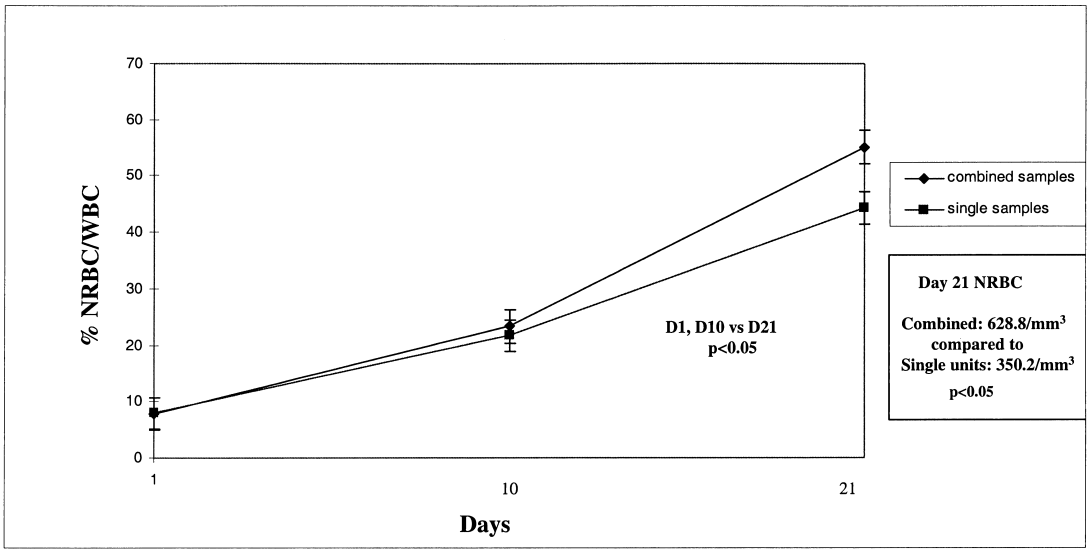

Fig. 2. Percentage of nucleated red blood cells per W.B.C following storage at 4° C for 21 days.

a survivable marrow transplant (7). From our previous studies on mixed lymphocyte cultures it appears that the transplant of stored cord blood may be without significant graft vs host disease by day 14 of storage at 4°C (6). However, there may be a significant drop off of essential cytokines by day 21, slightly decreasing the number of blast cell colonies. Combining multiple units of cord blood could also provide adequate numbers of stem cells for "megatherapy" (11, 12, 13, 14). The significance of the temperature, particularly the normoblast increase at which the cord blood was stored is being explored, however, it may be related to the primordial nature of the most primitive cells which we call "Berashis" cells (15).

Personal communication

From personal communications (official report is unavailable), we understand that 6×10^9 cord blood mononuclear cells were utilized to treat a patient involved in a recent nuclear accident in Tokaimura, Japan. While leaning over the container that had the nuclear reaction the patient received a lethal dose of 600–800 Rad. He initially had a good response to the cord blood cells and survived the immediate effect of irradiation, but now has evidence of chronic radiation damage to his head and chest. To our knowledge, this is the first time that cord blood was used for a patient exposed to a nuclear accident.

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