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Pre-clinical development of cord blood-derived progenitor cell graft expanded *ex vivo* with cytokines and the polyamine copper chelator tetraethylenepentamine

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Background

We have previously demonstrated that the copper chelator tetraethylenepentamine (TEPA) enables preferential expansion of early hematopoietic progenitor cells ($CD34^+ CD38^-$, $CD34^+ CD38^- Lin^-$) in human umbilical cord blood (CB)-derived $CD34^+$ cell cultures. This study extends our previous findings that copper chelation can modulate the balance between self-renewal and differentiation of hematopoietic progenitor cells.

Methods

In the present study we established a clinically applicative protocol for large-scale ex vivo *expansion of CB-derived progenitors. Briefly, $CD133^+$ cells, purified from CB using Miltenyi Biotec's (Bergisch Gladbach, Germany) CliniMACS separation device and the anti-CD133 reagent, were cultured for 3 weeks in a clinical-grade closed culture bag system, using the chelator-based technology in combination with early-acting cytokines (SCF, thrombopoietin, IL-6 and FLT-3 ligand). This protocol was evaluated using frozen units derived from accredited cord blood banks.*

Results

Following 3 weeks of expansion under large-scale culture conditions that were suitable for clinical manufacturing, the median output value of $CD34^+$ cells increase by 89-fold, $CD34^+ CD38^-$ increase by 30-fold and CFU cells (CFUc) by 172-fold over the input value. Transplantation into sublethally irradiated non-obese diabetic (NOD/SCID) mice indicated that the engraftment potential of the ex vivo *expanded $CD133^+$ cells was significantly superior to that of unexpanded cells: $60 \pm 5.5\%$ vs. $21 \pm 3.5\%$ $CD45^+$ cells, $P = 0.001$, and $11 \pm 1.8\%$ vs. $4 \pm 0.68\%$ $CD45^+ CD34^+$ cells, $P = 0.012$, $n = 32$, respectively.*

Discussion

Based on these large-scale experiments, the chelator-based ex vivo *expansion technology is currently being tested in a phase 1 clinical trial in patients undergoing CB transplantation for hematological malignancies.*

Keywords

ex vivo *large-scale expansion, pre-clinical development, tetraethylenepentamine.*

Introduction

Cord blood (CB) is a valuable source of stem cells. Transplanted CB hematopoietic stem/progenitors cells (HPC) can treat malignant and non-malignant disorders [1–3]. However, the major clinical limitation of CB transplantation is the low number of HPC in comparison with mobilized peripheral blood or BM grafts. This limitation may explain the slower time to engraftment and higher rate of engraftment failure following CB transplantation [4,5]. To overcome this limitation, *ex vivo* expansion of CB progenitors with a cocktail of growth factors has been attempted [6]. It was shown that a

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combination of early- and late-acting cytokines, including SCF, thrombopoietin (TPO), G-CSF and IL-3, resulted in only a marginal-fold expansion of late ($CD34^+$) and early ($CD34^+CD38^-$) progenitor cells, probably due the fact that the late-acting cytokines drive the cultures mainly toward accelerated differentiation [7–9]. On the other hand, cultures with only early-acting cytokines (SCF, TPO, IL-6 and FLT-3 ligand) resulted in better and prolonged expansion of both late and early progenitors [10,11], which are important for short-term early trilineage engraftment [12–14].

We have previously demonstrated that short-term (3 weeks) treatment with the polyamine copper chelator tetraethylenepentamine (TEPA) augmented the long-term expansion potential of CB-derived progenitor cells [15]. During the treatment period, TEPA inhibited the onset of cytokine-driven differentiation of early progenitor cells, resulting in a robust accumulation of $CD34^+CD38^-$ and $CD34^+Lin^-$ cells, with no effect on proliferation and differentiation of more mature committed cells [$CD34^+Lin^+$ and CFU culture (CFUc)] [16]. These results strongly suggest that TEPA supports the self-renewal division cycle without compromising differentiation capacity of hematopoietic stem cells.

In view of these results, the TEPA-based expansion procedure was adapted to comply with current good manufacturing practice (cGMP) standards required for clinical trials. In the present study we describe the development of a clinical-scale procedure using Miltenyi Biotec's (Bergisch Gladbach, Germany) CliniMACS separation device, and the anti-CD133 reagent for progenitor cell enrichment and 3-week expansion in culture bags, using the chelator-based technology with an early-acting cytokine cocktail (FLT-3 ligand, IL-6, TPO, SCF).

Methods

CB samples

Cells were obtained from neonatal umbilical cord blood after normal full-term delivery (informed consent was given). Samples were collected and frozen in our laboratories according to Rubinstein *et al.* [17] within 24 h postpartum, or kindly provided by the New York Blood Bank (New York, NY) and the Duke University Medical Center Cord Blood Bank (Durham, NC).

Thawing procedure and CliniMACS separation of $CD133^+$ and $CD34^+$ cells

The cells were thawed by doubling the volume of the blood sample in 2.5% dextran (Sigma, St Louis, MO) and 1.25% HSA (Bayer Co., Elkhart, IN). Prior to centrifuging an additional 40 mL of 10% dextran was added.

The cells were resuspended in 40 mL of 2.5% dextran/1.25% HSA and then filled to 100 mL with PBS (Biological Industries, Beit-HaEmek, Israel) containing 0.4% sodium citrate solution (Baxter HealthCare Co., Deerfield, IL) and 1% HSA. The pellet was incubated with 0.15% w/v intravenous immunoglobulin (IvIg; Omrix Biopharmaceuticals, Nes-Ziona, Israel) for 10 min at room temperature before centrifugation, and then resuspended in PBS containing 0.4% sodium citrate solution and 1% HAS, and 0.25 mg/mL recombinant human deoxyribonuclease (rHu-Dnase) added. Subsequently, the cells were labeled with Miltenyi's anti-CD133 (clone 1) or anti-CD34 CliniMACS reagent (Miltenyi Biotec) and separated by CliniMACS (according to the manufacturer's instructions). Following separation, cells were stained with trypan blue, counted, assayed for CFUc and immunophenotyped to determine purity.

Purity determination of CD34– and CD133–enriched cell fractions

The cells were washed with a PBS solution containing 1% BSA (PBS/1%BSA), and stained (at 4°C for 30 min) with FITC-conjugated anti-CD45 (Becton Dickinson, San Jose, CA) and either PE-conjugated anti-CD34 (DAKO, Glostrup, Denmark) or PE anti-CD133 (Miltenyi Biotec) Ab. In addition, the percentage of cells exhibiting both the CD133 and CD34 markers was measured by staining the cells with FITC–anti-CD34 (IQ Products, Groningen, the Netherlands) and PE–anti-CD133 (Miltenyi Biotec) Ab. The cells were then washed in the above buffer and analyzed using a FACScalibur® flow cytometer (Becton Dickinson, Immunofluorometry Systems, Mountain View, CA). The cells were passed at a rate of up to 1000 cells/second, using a 488-nm argon laser beam as the light source for excitation. Emission of 5000 cells was measured using logarithmic amplification, and analyzed using the CellQuest software (Becton Dickinson). Cells stained with FITC- and PE-conjugated isotype control Ab were used to determine background fluorescence.

Cell counting

The number of total nucleated cells (TNC) was determined by diluting the cells 1:2 with trypan blue and differentially counting viable and dead cells using a hemocytometer under an upright microscope at 100 × magnification.

Assay for CFUc

Cells, 1000 ($CD34^+$ or $CD133^+$) before culture and 1500 following culture, were added per 3 mL semisolid minimal essential alpha medium (MEMα), containing methylcellulose (Sigma), 30% FCS, 1% BSA, 1×10^{-5} M β-mercaptoethanol (Sigma), 1 mM glutamine (Biological Industries), 2 IU/mL erythropoietin (Eprex, Cilag AG Int., Schaffhausen, Switzerland), SCF and IL-3, both at 20 ng/mL, G-CSF and GM-CSF, both at 10 ng/mL (Perpo Tech Inc., Rocky Hill, NJ), and 2 μm hemin (Sigma). Following stirring, the mixture was divided into two 35-mm dishes and incubated for 14 days at 37°C in a humidified atmosphere of 5% CO_2 in air. At the end of the incubation period, colonies (both myeloid and erythroid) were counted under an inverted microscope at 40 × magnification. CFUc content was calculated as the following: number of scored colonies per two dishes × total cell number/1500 or 1000. The number of cells was determined by multiplying the number of cells/mL by the culture volume. CFUc frequency was calculated as number of colonies divided by the number of cells seeded.

Ex vivo expansion

Purified $CD34^+$ or $CD133^+$ cells were cultured at 1×10^4 cells/mL in MEMα and 10% FCS (Biological Industries) containing the following human recombinant cytokines: TPO, IL-6, FLT-3 ligand and SCF, each at a final concentration of 50 ng/mL (Pepro Tech Inc.), and 5 μm TEPA (Aldrich, Milwaukee, WI). VueLife Teflon PEP culture bags (American Fluoroseal Co., Gaithersburg, MD) were used: 72-mL bags were used for up to 20×10^4 initiating cells, and 270-mL bags were used for up to $20-70 \times 10^4$ cells. The cultures were incubated for 3 weeks (unless otherwise stated) at 37°C in a humidified atmosphere of 5% CO_2 in air. Cultures were topped weekly with the same volume of fresh medium, FCS, cytokines and TEPA. At the termination of the experiment, cells were counted following staining with trypan blue, assayed for CFUc and immunophenotyped for surface antigens.

Surface antigen analysis of cultured cells

The cells were washed with PBS/1%BSA and stained (at 4°C for 30 min) with both FITC–anti-CD45 and PE–anti-CD34 (both from DAKO) Ab for determination of $CD34^+$ cells, with FITC–anti-CD38 and PE–anti-CD34 for determination of $CD34^+CD38^-$ cells and with FITC–anti-CD45 and PE–Ab to lineage specific antigens (Becton Dickinson). The cells were then washed and analyzed as described above.

Clinical grade reagents

During the development phase, the research grade ingredients were replaced by clinical grade ingredients as follows.

Thawing procedure and CliniMACS separation: Gentran-40, a ready-made 10% w/v dextran solution, HSA and IvIg (all from Baxter), Dnase (Genentech Inc., San Francisco, CA) and PBS (Hyclone, Logan, UT). CFUc assay: MethoCult™, a methylcellulose-based medium (StemCell Technologies, Vancouver, Canada). *Ex vivo* expansion: MEMα and FCS (gamma-irradiated defined fetal bovine serum batch), from Hyclone.

TEPA was purchased from NovaSep (Boothwyn, PA). The cytokines TPO, IL-6, SCF and FLT-3 ligand were from R&D Systems (Minneapolis, MN). They are human recombinant cytokines from non-mammalian origin (derived from either *Escherichia coli* or Sf-21 cells). FLT-3 ligand and TPO were purified on affinity columns containing MAb. The cytokines were filtered through a 0.2-micron membrane, packaged under aseptic conditions and tested for endotoxin. Cytokine batches used in the study were tested for sterility by Charles River Laboratories (Rockville, MD) according to the ICH guideline *Viral safety evaluation of biotechnology products derived from cell lines of human or animal origin* adopted by the ICH Steering Committee (March 5, 1997) and the Code of Federal Regulations (April 1, 2003).

Reselection of cultured $CD34^+$ cells

To purify the $CD34^+$ cells, cultured cells were harvested and subjected to two cycles of immunomagnetic bead separation, using a MiniMACS CD34 progenitor cell isolation kit (Miltenyi Biotec) according to the manufacturer's recommendations. The purity of the $CD34^+$ population thus obtained was 90–98%, as evaluated by flow cytometry. The eluted cells were then counted and dually stained with PE–anti-CD34 and FITC–anti-CD38 Ab for determination of the percentage of $CD34^+CD38^-$

cells. $CD34^+$ cell content of the entire culture was calculated as follows: number of $CD34^+$ cells recovered following repurification × culture volume/volume of the portion of the culture subjected to repurification. Fold expansion of $CD34^+$ cells was calculated by dividing the $CD34^+$ cell content of the culture by the number of inoculated $CD34^+$ cells. The $CD34^+CD38^-$ cell content of the entire culture was calculated from the total $CD34^+$ cells. Fold expansion was calculated by dividing the total $CD34^+CD38^-$ cell number following culture by the number of inoculated cells.

Stability tests of the expanded graft

Following 3-week expansion, the cells were harvested, washed twice with PBS/EDTA–HSA solution, resuspended in transfusion solution (PBS/EDTA–HSA buffer) at $1-1.5 \times 10^6$ cells/mL and transferred (at least 30 mL) into a transfusion bag (Transfer bag-Terumo, Teruflex T-150, Tokyo, Japan). Closure clamps were used to prevent foaming. The bags were kept in a shipping container (Styrofoam) at 22 ± 4°C. Data loggers were put inside the container and its inner surface fastened for temperature monitoring. The bags were sampled to assess the number of viable cells and CFUc at 0, 6, 10 and 24 h.

Transplantation of human CB-derived $CD133^+$ cells into NOD/SCID mice

Each CB unit was frozen in two portions. $CD133^+$ cells purified from the first portion were cultured for 3 weeks with TEPA, as described above. The second portion of each unit was kept frozen until the day of transplantation (non-cultured cells). Mice were transplanted either with the progeny of 5×10^4 cultured $CD133^+$ cells or with 10×10^6 non-cultured mononuclear cells. Control mice were injected with medium only.

NOD/SCID mice, aged 10–11 weeks, bred and maintained at the Department of Immunology, the Weizmann Institute of Science, Rehovot, Israel, were injected intravenously with the above cells 1 day after they had been irradiated at 375 cGy.

The mice were killed 4 weeks post transplantation; BM was collected from both femurs and tibiae. The BM cells were washed in PBS/1%BSA and stained (at 4°C for 30 min) with PE-conjugated Ab to human CD45 (DAKO) and FITC-conjugated Ab to human CD34 (IQ Products), CD41, CD61, glycophorin A (DAKO), CD14, CD15, CD33 and CD19 (Becton Dickinson). Following incubation, the suspension was treated with FACS lysing solution (Becton Dickinson) to remove red blood cells, washed in PBS/1%BSA and analyzed by flow cytometry, as described above.

Calculations

Ex vivo expansion of TNC, CD34, $CD34^+CD38^-$ cells and CFUc are reported either as total numbers (number of cells per mL multiplied by the final culture volume) or as fold-expansion (total numbers divided by initial seeding cell number).

Statistics

The following statistical tests were used. The non-parametric test (Wilcoxon rank test) was applied for testing differences between the study groups for quantitative parameters. The data was analyzed using the SAS software (SAS version 8.2; SAS Institute Inc., Cary, NC).

Results

Progenitor cell purification

As a first step toward large-scale experiments and a clinical trial, we replaced the research-grade CD34-based separation device with the clinical grade CD133-based device. For this purpose, we compared the anti-CD34 and anti-CD133 CliniMACS reagents, using the CliniMACS separation device, with respect to the yield (number of cells) (Figure 1a) and purity (percent of $CD34^+$ or $CD133^+$ cells) (Figure 1b). The results indicated no statistically significant difference. Most ($>90\%$) of the cells in the enriched populations were double positive for both CD133 and CD34. A representative FACS analysis is shown in Figure 1c. A comparison of the fractions with respect to their CFUc frequency also produced similar results, $0.2 \pm 0.1\%$ and $0.12 \pm 0.07\%$, respectively.

Clinical-scale *ex vivo* expansion

To optimize the duration of the expansion procedure, we compared 2- vs. 3-week cultures. Cultures were initiated with 1×10^4 cells/mL purified by the CliniMACS utilizing the anti-CD133 reagent. The cells were grown in 290-mL culture bags (initial culture volume/bag, 25 mL) in alpha medium supplemented with FCS, a combination of four cytokines (SCF, TPO, IL-6 and FLT-3 ligand, 50 ng/mL each) and 5 μm TEPA. The cultures were topped up weekly with an equal volume of fresh medium.

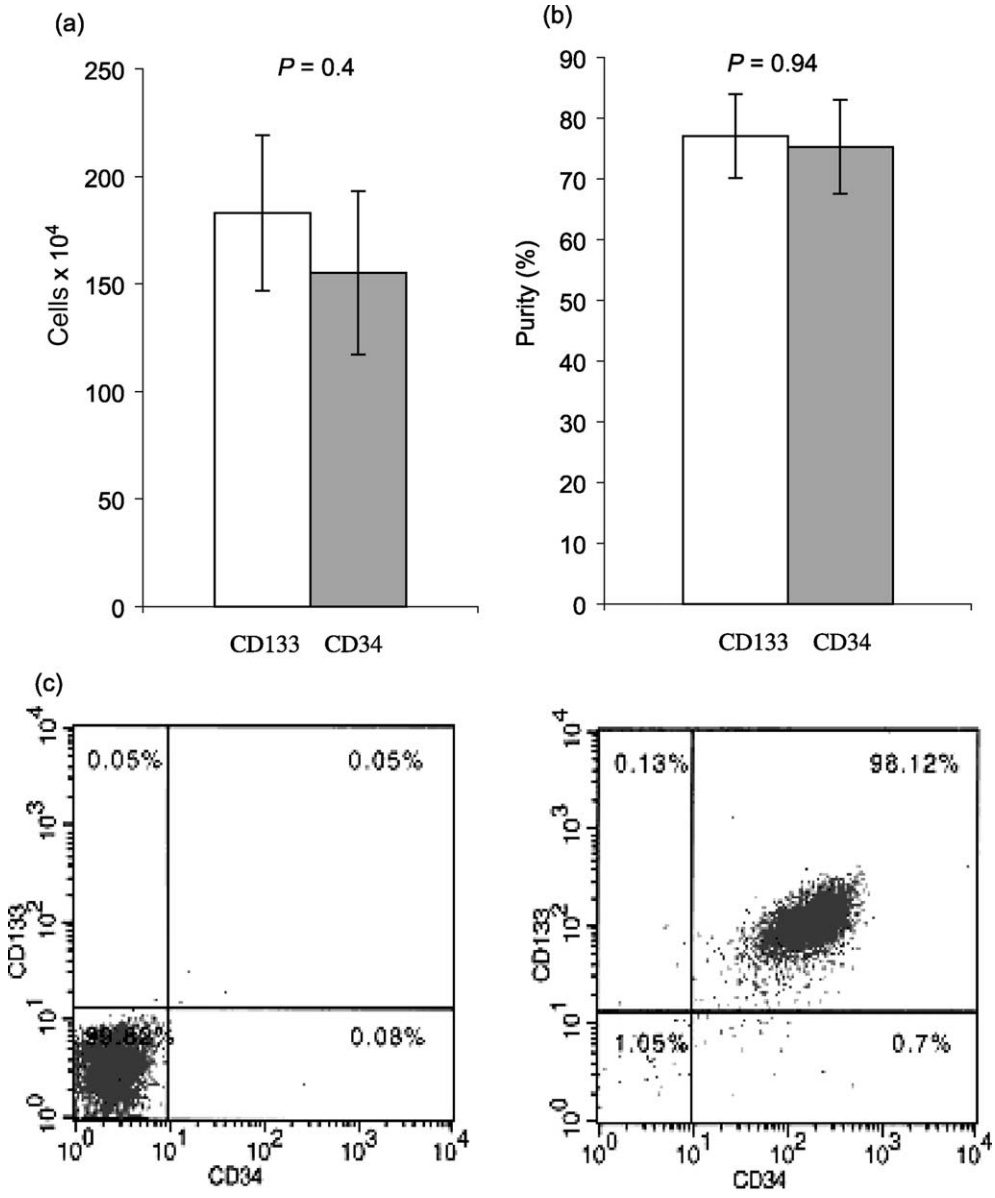

Figure 1. *Comparison between anti-CD133 and anti-CD34 CliniMACS enrichment reagents. Frozen CB units (*n = 6*) were thawed, divided into two equal portions and enriched for progenitor cells using anti-CD133 or anti-CD34$^+$ reagents and CliniMACS separation device. Cell yield (a) was determined by counting the number of viable cells in the positive fraction. Purity (b) was determined by FACS analysis of double stained cells with PE–anti-CD45 and either anti-CD34 or FITC–anti-CD133 Ab. A representative FACS analysis dot-plot of CD133-enriched cells is shown in (c). The eluted cells were double stained with isotype controls (left panel) or with both PE–anti-CD133 and FITC–anti-CD34 Ab (right panel). The percentage of cells in each quadrant is indicated.*

Figure 2 shows that the cumulative numbers ($\times 10^6$) of TNC, CD34$^+$ and CD34$^+$CD38$^-$ cells were significantly higher following 3-week expansion compared with 2 weeks: 84 ± 7 vs. 34 ± 3, 22 ± 2 vs. 11 ± 2, and 5.4 ± 1 vs. 2.6 ± 0.4, respectively. Only limited expansion was observed following the first week of culturing (data not shown).

We then compared the expansion potential of cells purified from the same CB unit with either the anti-CD133$^+$ or the anti-CD34$^+$ reagents. The cultures ($n = 4$) were initiated with 2.5×10^5 cells and grown for 3 weeks. The yield of TNC was $1065 \pm 124 \times 10^5$ and $760 \pm 75 \times 10^5$ ($P = 0.19$), CFUc 81 ± 9 and $83 \pm 11 \times 10^5$ ($P = 0.66$), CD34$^+$ cells $43 \pm 7 \times 10^5$ and $39 \pm 9 \times 10^5$

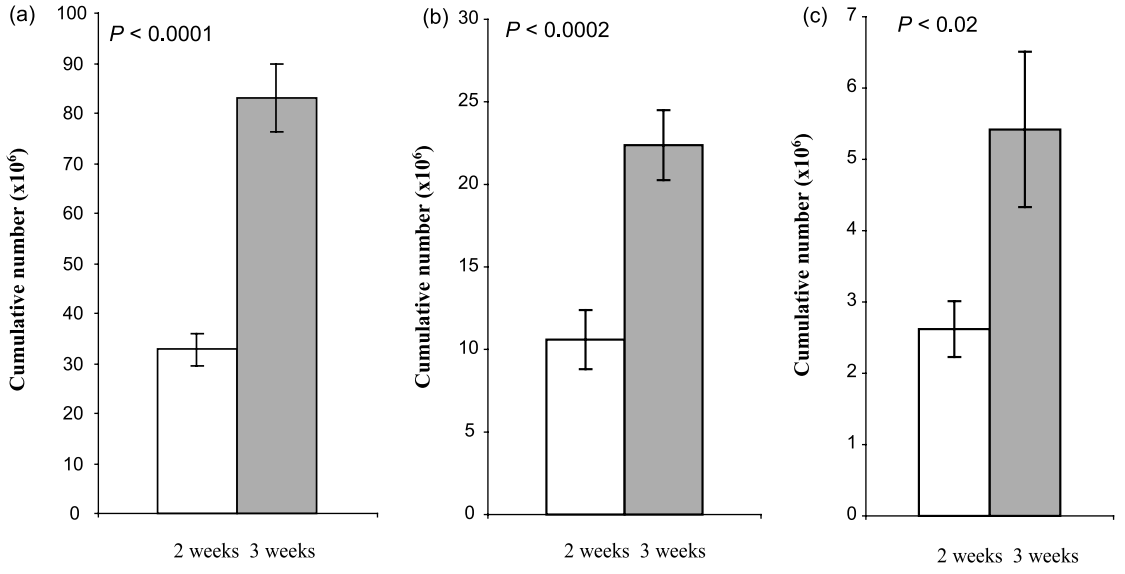

***Figure 2**. Optimization of the expansion duration. Cultures* (n = *18) were initiated with purified CD133$^+$ cells. TNC (a), CD34$^+$ (b) and CD34$^+$CD38$^-$ (c) cells were determined after 2 and 3 weeks. Cumulative numbers were calculated as described in the Methods.*

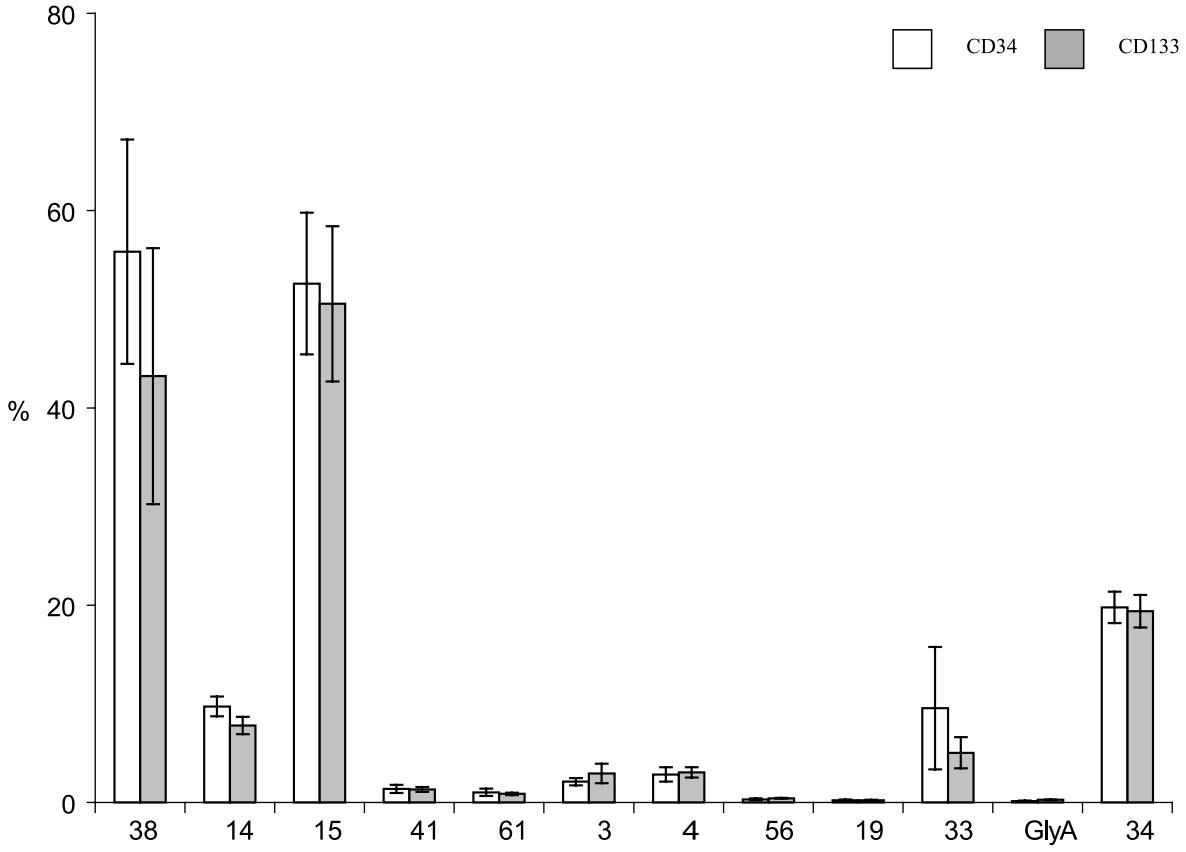

***Figure 3**. Phenotype analysis of 3-week cultures. Cultures were initiated with either CD133$^+$ or CD34$^+$ cells. After 3 weeks cultured cells* (n = *18) were double stained with PE– anti-CD45 and FITC– anti-lineage specific Ab and analyzed by FACS. The percentages of cells expressing CD38, myeloid (CD14, CD15, CD33), lymphoid (CD3, CD4, CD19, CD56), erythroid, (GlyA) and megakaryocytic (CD41, CD61) antigens as well as that expressing the progenitor cell antigen (CD34) are shown.*

($P = 0.89$), $CD34^+CD38^-$ cells $12 \pm 3.6 \times 10^5$ and $5.6 \pm 1.3 \times 10^5$ ($P = 0.11$), in cultures initiated with $CD133^+$ cells and with $CD34^+$ cells, respectively. In this set of experiments, $CD34^+$ and $CD34^+CD38^-$ cells were determined following affinity reselection of $CD34^+$ cells, as described in the Methods. Additional immunophenotyping indicated similar proportions of cells expressing myeloid, lymphoid or megakaryocytic phenotype in cultures initiated either with $CD34^+$ or $CD133^+$ cells (Figure 3).

Evaluation of the expansion procedure

Based on the above-described experiments, we carried out a large-scale evaluation of the following expansion procedure. A 20% portion of a CB unit was thawed and progenitor cells were enriched by the CliniMACS anti-CD133 procedure. The purified cells were grown for 3 weeks in culture bags with cGMP components, including cytokines and TEPA. Of the frozen CB units studied, 18 were derived from accredited CB banks (Netcord Düsseldorf, Germany, and COBLT) and 4 units from the Gamida-Cell research-grade CB bank (Jerusalem, Israel). The results showed that the yield of viable cells was in the range of $17-35 \times 10^4$ and the purity 58–97%. The percentages of $CD34^+$ and $CD34^+CD38^-$ cells following 3-week expansion are shown in Figure 4.

The input numbers of $CD34^+$, $CD34^+CD38^-$ and CFU cells as well as the output numbers following 3 weeks expansion are shown in Table 1. The median output value of $CD34^+$ cells increased by 89-fold, $CD34^+CD38^-$ increased by 30-fold and CFUc by 172-fold over the input values.

We then determined the expansion efficacy with regard to the number of cells of different subtypes available for transplantation. For this purpose, the number of cells in the expanded product (derived from 20% of the CB unit) and the number of cells in the non-manipulated 80% portion, were combined and compared with the number of cells in the whole (100%) non-manipulated unit (Table 2). Statistical analysis of the data demonstrated that the numbers of CFUc, $CD34^+$ and $CD34^+CD38^-$ cells in a graft also containing expanded cells were significantly higher than in a non-manipulated graft ($P < 0.025$), whereas the total nuclear cell numbers were comparable ($P = 0.4$).

Stability of the expanded graft

To prepare the expanded cell product for transplantation, the cells are resuspended in infusion buffer and transferred into a transfusion bag. Since there may be a delay of several hours between the completion of the manufacturing process and the infusion of the cells into the patient, we conducted a 24-h stability study as described in the Methods. Cell samples were taken immediately after inoculation of the cells into the transfusion bags, and 6, 10 and 24 h thereafter. The results shown in Table 3 demonstrate that the numbers of viable cells and CFUc during the 24 h were statistically comparable.

NOD/SCID engraftment potential of the expanded graft

The marrow-repopulating ability of the expanded cells in NOD/SCID mice was compared with that of non-

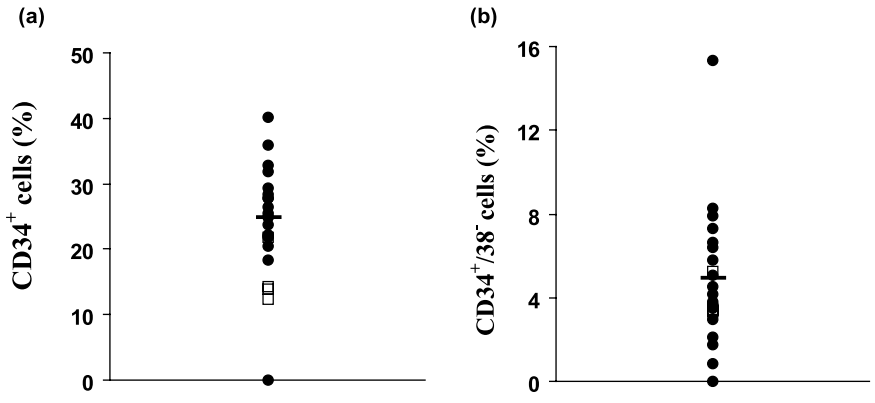

Figure 4. *Large-scale evaluation of the 3-week expansion procedure. Cultures were initiated with $CD133^+$-enriched cells following separation on CliniMACS of 22 CB units [four research-grade units (squares) and 18 clinical-grade units (circles)]. After 3 weeks, the cultured cells were double stained with PE–anti-CD45 and FITC–anti-CD34 (a) or FITC–anti-CD34 and PE–anti-CD38 (b). Percentages of $CD34^+$ and $CD34^+CD38^-$ cells were determined by FACS analysis.*

Table 1. Expansion of cells following 3-week culture

	Input			Output			Median-fold increase
	Range	Median	Mean ± SE	Range	Median	Mean ± SE	
TNC × 10^7				2–20	12	12 ± 3	
$CD34^+$ × 10^4	17–35	25	25 ± 2	131–3061	2224	2120 ± 556	89
$CD34^+CD38^-$ × 10^4	2–17	11	10 ± 3	33–803	335	361 ± 103	30.5
CFU × 10^4	2–7	3	4 ± 1	136–1100	517	537 ± 144	172

The cultures were initiated with $CD133^+$ cells derived from a 20% portion of a CB unit. At the initiation of the cultures (input) and after 3 weeks (output), cells were analyzed for the indicated parameters. Median-fold increase was calculated by dividing the median output by the median input values.

expanded cells, both derived from the same CB unit. Mice were concomitantly transplanted with either 10×10^6 non-expanded mononuclear cells (containing 5×10^4 $CD133^+$ cells) or all the progeny of purified 5×10^4 $CD133^+$ cells following a 3-week large-scale expansion. In all eight experiments, mice injected with cultured cells contained a significantly higher percentage of total human ($CD45^+$) cells (Figure 5a and Table 4), and human progenitor ($CD45^+CD34^+$) cells (Figure 5b), compared with mice injected with non-cultured cells ($P = 0.001$ and $P = 0.012$, respectively). Calculated engraftment efficacy, i.e. the percentage of $CD45^+$ cells in mice transplanted with cultured cells divided by the percentage of $CD45^+$ cells in mice transplanted with non-cultured cells, ranged from 1.7 to 31.

Phenotype analysis demonstrated that the expanded cells maintained the potential to differentiate *in vivo* into various hematopoietic lineages, myeloid (CD14, CD15, CD33), megakaryocyte (CD41 and CD61), erythroid (glycophorin A) and B lymphoid (CD19). The engraftment of all assessed hematopoietic lineages was significantly higher in mice transplanted with expanded cells compared with non-cultured cells (Figure 5b).

Discussion

Umbilical CB has been used successfully as a source of hematopoietic stem cells in allogeneic stem cell transplantation. Advantages of using CB include reduced susceptibility to post-transplant infections and to GvHD, as well as greater availability of a donor. The major limitation of using CB is related to the low cell dose in CB and possibly to some intrinsic properties of CB cells [18]. *Ex vivo* expansion is a strategy to increase the number of cells available for transplantation. Two general protocols suitable for clinical application have been published [19–21]. The first protocol comprises a two-step culture system

Table 2. Number of specific cells available for infusion in the non-expanded vs. expanded graft

No. cells	Treatment	Median	Mean ± SE	*P*-value	Expansion efficacy***
TNC × 10^7	Non-expanded*	76.3	71.8 ± 5.8		
	Expanded**	74.3	69.4 ± 6.6	0.4	0.97
$CD34^+$ cells × 10^4	Non-expanded	124.5	118.3 ± 18		
	Expanded	2345.0	2220 ± 559	0.0025	19
$CD34^+CD38^-$ cells × 10^4	Non-expanded	53.5	46.8 ± − 12.7		
	Expanded	378.0	398.3 ± − 112.2	0.025	7
CFUc × 10^4	Non-expanded*	15.0	17.5 ± 4.3		
	Expanded	528.0	550.7 ± 146	0.009	35

Cells in each CB unit were frozen in two portions of 80% and 20%. The 20% portion was thawed, counted and the CD133 cells purified and cultured for 3 weeks as described in the Methods. The numbers present the cells of the various subsets calculated for the 100% non-expanded cells of the CB unit (*) and a mixture of the 80% non-expanded plus the culture output of the 20% portion (**).

****Calculated by dividing the median expanded cell graft values by the median non-expanded graft values.*

Table 3. Stability of the expanded graft

Exp. #	0 h	6 h	10 h	24 h
(a) No. viable cells $\times 10^4$/mL				
1	77 ± 0.6	73 ± 0.3	77 ± 3.9	91 ± 3.8
2	64 ± 11	50 ± 3.1	54 ± 2.0	37 ± 6.9
3	104 ± 5.2	106 ± 4.8	96 ± 12.1	102 ± 6.9
(b) CFU $\times 10^4$/mL				
1	3.3 ± 0.2	3.6 ± 0.1	4.0 ± 0.4	4.2 ± 0.1
2	2.1 ± 0.3	3.2 ± 0.2	1.6 ± 0.2	2.0 ± 0.4
3	6.9 ± 0.4	7.0 ± 0.4	6.6 ± 0.7	7.3 ± 0.9

Following 3-week expansion, the cells were harvested, washed and transferred into bags as detailed in the Methods. The bags were kept in a shipping container at $22 \pm 4^{\circ}$C and sampled to assess the number of viable cells (a) and CFUc (b) at time 0, 6, 10 and 24 h.

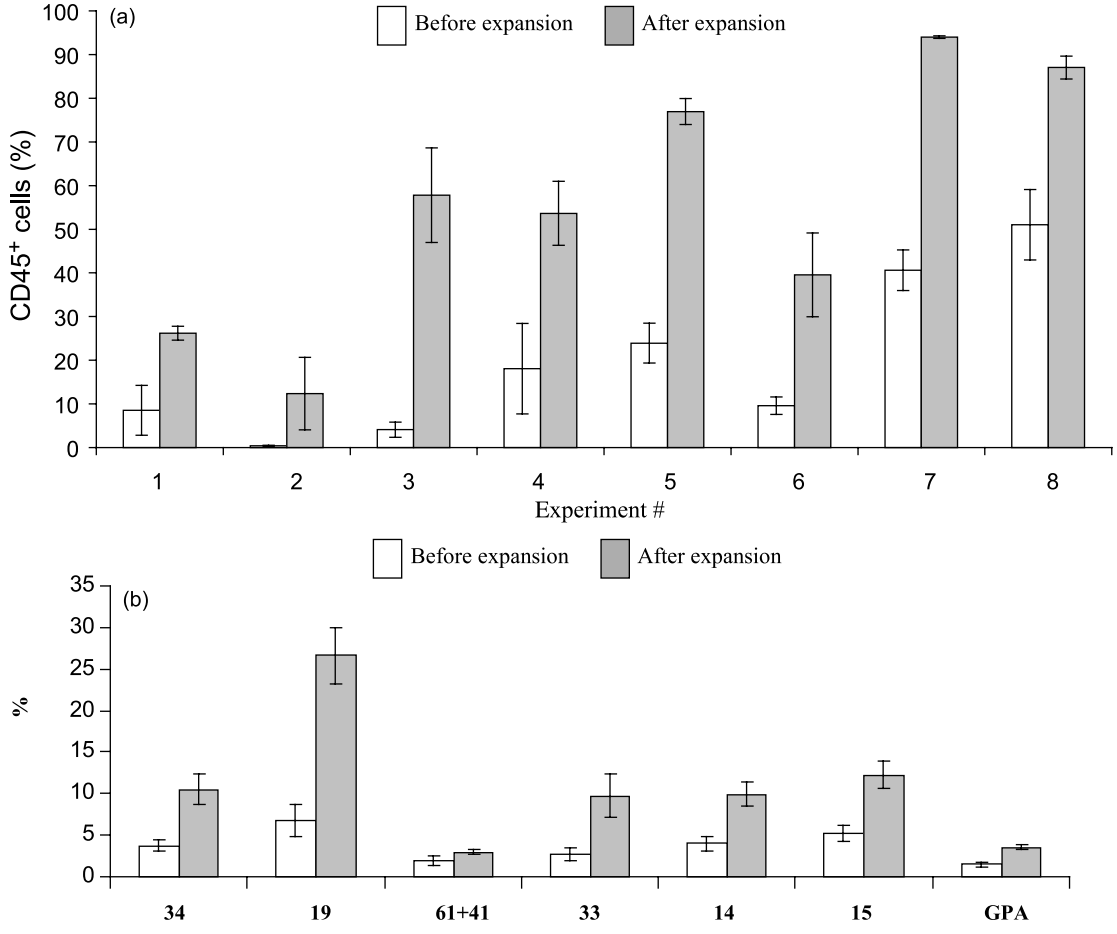

Figure 5. *Engraftment of clinical-grade cultured cells in NOD/SCID mice. Mice (3–5 per experimental group) were transplanted with all the progeny of 5×10^4 CD133$^+$ cells after 3 weeks expansion or with the equivalent fraction of MNC before expansion (10×10^6 cells), both derived from the same CB units. Four weeks after transplantation, human cell engraftment was evaluated by FACS analysis of CD45$^+$ cells (a). Progenitor cells (CD34$^+$) and lineage-specific differentiated cells were evaluated by FACS analysis of cells double stained with PE–anti-human CD45 and FITC–anti-human lineage antigens (b). The data in (a) present the mean $\pm$ SE of each experiment of eight (#1–8) consecutive experiments. The data in (b) present the mean $\pm$ SE of four experiments. GPA = GlycophorinA.*

Table 4. Engraftment of human cells in SCID mice BM

Exp. #	% CD45$^+$ human cells in mouse BM 4 weeks after transplantation													
	Non-cultured cells						Cultured cells							
	Mouse 1	Mouse 2	Mouse 3	Mouse 4	Mouse 5	Average	SE	Mouse 1	Mouse 2	Mouse 3	Mouse 4	Mouse 5	Average	SE
1	20.0	3.6	2.1	–	–	8.6	5.7	28.9	23.3	26.3	–	–	26.2	1.6
2	0.2	0.6	0.4	–	–	0.4	0.1	28.9	5.1	3.1	–	–	12.4	8.3
3	7.5	2.9	1.9	–	–	4.1	1.7	63.5	36.8	73.0	–	–	57.8	10.8
4	37.6	2.4	14.2	–	–	18.1	10.4	61.0	60.9	39.0	–	–	53.6	7.3
5	8.7	26.7	19.0	33.7	31.6	23.9	4.6	78.2	69.4	83.8	76.4	D	77.0	3.0
6	9.0	11.7	14.5	10.2	2.5	9.6	2.0	60.3	14.4	37.5	45.9	D	39.5	9.6
7	46.1	28.4	49.3	38.6	D	40.6	4.7	94.6	94.3	93.2	93.8	D	94.0	0.3
8	43.2	67.5	32.3	61.1	D	51.0	8.1	77.5	86.6	91.8	87.7	91.6	87.0	2.6

The table demonstrates the raw data of the experiments presented in Figure 5a.
D = dead; – = not done.

initiated with CB-derived CD34$^+$ cells. The cells were cultured in Teflon culture bags supplemented with defined medium containing SCF, G-CSF and TPO [9]. The second protocol comprises an automated continuous perfusion culture device developed by Aastrom Biosciences [21]. In this system cultures are initiated with CB-derived mononuclear cells and expanded for 12 days in media supplemented with FBS, horse serum, PIXY321, FLT-3 ligand and erythropoietin [21]. The duration of both clinical applicative protocols is 12–14 days and in both protocols the cultures are supplemented with a mixture of early- and late-acting cytokines. These conditions accelerate cell proliferation and differentiation, resulting in increased expansion of TNC and further maturation of myeloid precursors. Clinical studies performed using these protocols demonstrated that clinical-scale expansion of CB is feasible, and that administration of these cells is well tolerated [19–21].

Our current study presents an applicable protocol for clinical-scale *ex vivo* expansion of CB-derived CD133$^+$ progenitor cells in the presence of early-acting cytokines and the copper chelator TEPA. As opposed to the previously reported expansion protocols, it appears that the present culture conditions enable better expansion of less differentiated cell subsets.

Due to regulatory and proprietary concerns, we utilized the Miltenyi Biotec's CliniMACS apparatus [22] and the anti-CD133 CliniMACS reagent, both of them clinically approved, replacing the research-grade MiniMACS CD34 progenitor cell isolation kit. The CD133 antigen is a novel marker for stem/progenitor cells. Phenotypic and functional studies indicated that the CD133-enriched population could serve as an alternative to CD34$^+$ cell selection and engraftment purposes [23–25]. Furthermore, CD133$^+$ selected cells have already been used in clinical transplantation settings without any safety impairment [26]. In our study, we demonstrated similar yield and purity, crucial parameters for successful transplantation [22], using the CliniMACS instrument in combination with either the anti-CD34 or anti-CD133 reagent.

The duration of most expansion protocols for clinical application is between 10 and 14 days [19–21]. We evaluated the feasibility of the chelator technology to extend the expansion in order to maximize the number of early and late progenitor cells. To this end, we compared 2 vs. 3-week cultures and found that the latter was superior with respect to the yields of TNC, CD34$^+$ and

$CD34^+CD38^-$ cells (Figure 2). Although the technology enables longer expansion [16], it is not desirable in a clinical setting. Following a 3-week large-scale clinical grade expansion, the yield of early progenitor ($CD34^+CD38^-$) cells was higher in cultures initiated with $CD133^+$ cells ($12 \pm 3.6 \times 10^5$) than in cultures initiated with a similar number of $CD34^+$ cells ($5.6 \pm 1.3 \times 10^5$).

Using optimized clinically applicable conditions, e.g. CD133 cell enrichment, CliniMACS separation device and culture bags, we evaluated the procedure on 22 frozen CB units, 18 of which were obtained from accredited CB banks. In spite of the high variability among CB units [27], we demonstrated the efficacy of the procedure to expand early and late progenitor cells. In these experiments the median output value of $CD34^+$ cells increased by 89-fold, $CD34^+CD38^-$ increased by 30-fold and CFUc by 172-fold over the input values.

We then determined the expansion efficacy with regard to the number of cells of different subtypes available for transplantation. Since in clinical trials expanded cells will be given in addition to non-manipulated cells of the same unit, the efficacy depends on the portion of the unit taken for expansion. We calculated the efficacy based on the expanding 20% portion of the unit. The results show (Table 2) that the major contribution of the expanded product is in the numbers of early progenitor cells ($CD34^+CD38^-$) as well as that of late progenitor cells ($CD34^+$, CFUc). Numbers of TNC in the expanded graft were comparable to those in the non-expanded unit.

Finally, we demonstrated that our clinical-scale expanded cells successfully engrafted SCID mice. In eight consecutive experiments, the percentage of engrafted human progenitors as well as that of myeloid and lymphoid cells was significantly superior in mice transplanted with expanded cells to that in mice transplanted with non-expanded cells.

In summary, we describe a 3-week large-scale expansion procedure, utilizing a combination of copper chelator (TEPA) with early-acting cytokines (SCF, TPO, IL-6 and FLT-3 ligand), a clinically approved separation device and clinical grade reagents. Extensive research and development work demonstrated that FCS is suitable for the expansion process. As there is no regulation that prohibits the use of FCS in clinical trials in the USA, the use of a specific lot of FCS and its certificates of analysis were submitted to the FDA as part of Gamida Cell's investigational new drug (IND) application. The expansion procedure, evaluated using CB units derived from accredited CB banks, was demonstrated to produce a high yield of early progenitors with increased SCID engraftment potential. This novel strategy for *ex vivo* expansion of CB progenitors is currently under study in a phase 1 clinical trial.

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