ELSEVIER

EXPERIMENTAL HEMATOLOGY

Experimental Hematology 32 (2004) 547–555

Linear polyamine copper chelator tetraethylenepentamine augments long-term ex vivo expansion of cord blood-derived CD34$^+$ cells and increases their engraftment potential in NOD/SCID mice

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(Received 29 August 2003; revised 2 March 2004; accepted 9 March 2004)

Objective. **We previously demonstrated that cellular copper is involved in the regulation of proliferation and differentiation of hematopoietic progenitor cells. Modulation of cellular copper was achieved by supplementing the culture with a copper chelator that reduces cell copper content, or copper salts, which elevate the level of cellular copper. In the present study, we evaluated the effect of short-term (3-week) treatment with the copper chelator tetraethylenepentamine (TEPA) on short- and long-term (up to 11 weeks) ex vivo expansion of hematopoietic progenitors, as well as on their SCID engraftment potential.**

Materials and Methods. **Cord blood-derived purified CD34$^+$ cells were grown in liquid medium supplemented with the cytokines stem cell factor, thrombopoietin, Flt3 ligand, and IL-6, and the chelator TEPA for the first 3 weeks and then for up to 11 weeks with cytokines alone. Control cultures were supplemented with cytokines alone for the entire culture duration. Cultured cells were characterized by immunophenotyping and cloning (CFUc). Transplantability was assayed by injection of repurified CD34$^+$ cells into NOD/SCID mice.**

Results. **In the short term, TEPA supported increased percentages of early progenitors over control cultures incubated with cytokines alone (CD34$^+$CD38$^-$, $p = 0.001$ and CD34$^+$Lin$^-$, $p = 0.016$). In the long term, TEPA pretreated cultures showed prolonged expansion of CD34$^+$ cells ($p = 0.01$) and CFUc ($p = 0.002$) compared with that of untreated cultures. The SCID engraftment potential of CD34$^+$ cells repurified from the TEPA-treated cultures was higher compared with that of the control, i.e., only cytokine-treated cultures ($p = 0.03$).**

Conclusion. **TEPA enabled preferential proliferation of early progenitor cells with the phenotype CD34$^+$CD38$^-$ and CD34$^+$CD38$^-$ Lin$^-$ during the first weeks of culture, resulting in the observed increased long-term ex vivo expansion and engraftment capabilities.**

Several articles have suggested the causal role of copper (Cu) in modulating cell fate and differentiation in the hematopoietic system. Cu augmented the retinoic acid effect on the proliferation and differentiation of human myeloid leukemia HL-60 cells [1]. Induction of human erythroleukemia K562 cell differentiation was accompanied by a significant increase in cellular Cu content [2]. Lowering of cellular Cu by the Cu chelator tetraethylenetetramine (TETA) abolished 1,25-dihydroxyvitamin D_3 or phorbol 12-myristate 13-acetate induction of U937 cell differentiation [3]. Supplementation with Cu, but not with zinc (Zn) or iron (Fe), blocked the TETA-induced decline in cell Cu and reversed the TETA inhibitory effect. The most common clinical manifestations of Cu deficiency are anemia, neutropenia, and thrombocytopenia. These phenomena were reversed by administration of dietary Cu. A recent report described a patient with severe neutropenia and morphologic characteristics typical of myelodysplastic syndrome due to severe Cu deficiency that were fully resolved by Cu therapy [4]. Examination of the bone marrow (BM) of neutropenic, Cu-deficient patients revealed intact progenitors despite their maturation arrest

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0301-472X/04 $–see front matter.
doi: 10.1016/j.exphem.2004.03.002

and suggested the involvement of Cu in the regulation of hematopoiesis [5–10].

Using $CD34^+$ cell-enriched cultures, we previously demonstrated that cellular Cu is involved in the regulation of hematopoietic progenitor cell proliferation and differentiation [11]. In addition, we showed that Cu chelators such as tetraethylenepentamine (TEPA), at concentrations that moderately lower cellular Cu, delayed cell differentiation, whereas external addition of Cu salts or ceruloplasmin resulted in an elevated cell Cu content, as well as accelerated cell differentiation, regardless of the cytokines present in the culture medium [11].

In the present study, we demonstrate that 3-week treatment with TEPA results in enrichment of a cord blood-derived progenitor cell subpopulation that displays prolonged ex vivo expansion of $CD34^+$ cells and an enhanced capacity to repopulate NOD/SCID mice.

Materials and methods

Cord blood samples

After obtaining informed consent, cells were obtained from umbilical cord blood after normal full-term delivery. Samples were collected and frozen according to Rubinstein et al. [12] within 24 hours postpartum. Prior to use, the cells were thawed in Dextran buffer (Sigma, St. Louis, MO, USA) containing 2.5% human serum albumin (HSA; Bayer Corp. Elkhart, IN, USA). In order to avoid cell clumping, which interferes with MiniMACS $CD34^+$ cell enrichment of thawed cells and frequently results in low cell recovery, the cells were first preincubated for approximately 15 hours in α-minimal essential medium (MEMα) supplemented with 10% fetal calf serum (FCS) (both from Biological Industries, Beit-HaEmek, Israel). Following the preincubation, the cells were layered on a Ficoll-Hypaque gradient (1.077 g/mL; Sigma), and centrifuged at 800*g* for 30 minutes. The mononuclear cells in the interface layer were collected and washed three times in phosphate-buffered saline (PBS; Biological Industries) containing 0.5% HSA. To purify the $CD34^+$ cells, the mononuclear cell fraction was subjected to two cycles of immunomagnetic bead separation using a MiniMACS CD34 progenitor cell isolation kit (Miltenyi Biotec Bergisch, Gladbach, Germany), according to the manufacturer's recommendations. The purity of the $CD34^+$ population thus obtained was 95–98%, as evaluated by flow cytometry.

Ex vivo expansion

Purified $CD34^+$ cells were cultured in culture bags (American Fluoroseal Co., Gaithersburg, MD, USA) at a concentration of 1×10^4 cells/mL in MEMα/10% FCS containing the following human recombinant cytokines: thrombopoietin (TPO), interleukin-6 (IL-6), Flt-3 ligand, and stem cell factor (SCF), each at a final concentration of 50 ng/mL (Pepro Tech, Inc., Rocky Hill, NJ, USA), with or without 5 μM TEPA (Aldrich, Milwaukee, WI, USA) and incubated at 37°C in a humidified atmosphere of 5% CO_2 in air. Until week 3, the cultures were topped weekly with the same volume of fresh medium, TEPA, and growth factors. From week 3 and until termination of the experiments, the cultures were demi-depopulated weekly. Cells were counted after staining with trypan blue. At various time points, harvested cells were used to assay the content of colony-forming units in culture (CFU-C), enumeration of $CD34^+$ cells following reselection, and immunophenotype analysis. Cell morphology was determined on cytospin (Shandon, Pittsburgh, PA, USA) prepared smears stained with May-Grünwald/Giemsa solutions.

Assay for CFU-C

Mononuclear cells at 1500 per 3 mL were added to semisolid medium containing methylcellulose (Sigma), 30% FCS, 1% bovine serum albumin (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 Pepro Tech), and 2 μM hemin (Sigma). After stirring, the mixture was divided into two 35-mm dishes. The dishes were incubated for 14 days at 37°C in a humidified atmosphere of 5% CO_2 in air. At the end of the incubation period, myeloid and erythroid colonies were counted under an inverted microscope at 40× magnification. CFU-C content of the expansion culture was calculated as follows: number of scored colonies per two dishes × total mononuclear cell number/1500. Up to week 3, total mononuclear cells were determined by multiplying the number of cells per milliliter by the culture volume. From week 3 and on, the number of passages was also taken into account.

Surface antigen analysis

The cells were washed with a PBS solution containing 1% BSA and stained (at 4°C for 30 min) with fluorescein isothiocyanate (FITC)- or phycoerythrin (PE)-conjugated antibodies. The cells were washed in the previously described buffer and analyzed using a FACScalibur flow cytometer (Becton Dickinson, San Jose, CA, USA). 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 10^4 cells was measured using logarithmic amplification and analyzed using the CellQuest software (Becton Dickinson). Cells stained with FITC- and PE-conjugated isotype control antibodies were used to determine background fluorescence.

Determination of $CD34^+$ cell content after expansion

The content of $CD34^+$ cells was determined from a purified, reselected fraction using the MiniMACS CD34 progenitor cell isolation kit (Miltenyi Biotec), according to the manufacturer's recommendations. In brief, mononuclear cells derived from one portion of the culture were subjected to two cycles of immunomagnetic bead separation. The purity of the $CD34^+$ population thus obtained was 95–98%, as evaluated by flow cytometry. $CD34^+$ 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. Up to week 3, the cultures were topped weekly with fresh medium. Therefore, the culture volume was measured directly. From week 3 and on, the culture volume was calculated by multiplying the actual volume with the number of passages. Fold expansion was calculated by dividing the $CD34^+$ cell content of the culture by the number of inoculated $CD34^+$ cells. Fluorescence-activated cell sorting (FACS) analysis of cells from 8-week cultures showed that the forward light scatter (FSC-H) and side light scatter (SSC-H) of the repurified $CD34^+$ cells were similar to those of the $CD34^+$ cells before culture. Giemsa staining showed that the morphology of the cells was identical to that of freshly purified $CD34^+$ cells (data not shown).

Determination of early $CD34^+$ cell subsets

The percentages of early $CD34^+$ cell subsets were determined from the repurified $CD34^+$ cell fraction. Cells were dually stained with PE anti-CD34 and FITC anti-CD38 for determination of $CD34^+CD38^-$ cells and with PE anti-CD34 antibodies and a mixture of FITC-conjugated antibodies against differentiation antigens (CD38, CD33, CD14, CD15, CD3, CD61, CD19) for determination of $CD34^+Lin^-$ cells. Antibodies to CD34, CD38, and CD61 were purchased from DAKO (Glostrup, Denmark) and antibodies to CD33, CD14, CD15, CD3, and CD19 from Becton Dickinson.

FACS analysis results of these subsets are given as percentage of $CD34^+$ cells. Absolute number of $CD34^+CD38^-$ and $CD34^+Lin^-$ cells in the culture was calculated from the total number of $CD34^+$ cells recovered following the repurification step.

Transplantation of human cord blood-derived $CD34^+$ cells into NOD/SCID mice

Each cord blood unit was frozen in two portions. $CD34^+$ cells purified from the first portion of several units were pooled and cultured for 5 weeks with and without TEPA, as described earlier. The second portion of each cord blood unit was kept frozen (noncultured cells) until the day of transplantation, when the $CD34^+$ cells were purified from both the cultured and the noncultured cells. NOD/SCID mice were bred and maintained at the Department of Immunology, Weizmann Institute of Science, Rehovot, Israel. Purified $CD34^+$ cells together with 2×10^6 irradiated supportive cells taken from the $CD34^-$ fraction of the noncultured cells were injected intravenously into mice (age 10–11 weeks) 1 day after they had been irradiated at 375 cGy. A quantity of 2×10^6 irradiated $CD34^+$ cells was injected into the negative control mice.

The mice were sacrificed 8 weeks posttransplantation; BM was collected from both femurs and tibiae. BM cells were washed in PBS containing 1% BSA and stained (at 4°C for 30 minutes) with PE-conjugated anti-human CD45 (DAKO) and FITC-conjugated anti-human CD34 antibodies (IQ Products, Groningen, the Netherlands) or FITC-conjugated anti-lineage specific antibodies (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 earlier. The BM of control mice showed that less than 0.12% of cells stained positive with anti-human CD45 antibodies.

Statistical analyses

The nonparametric test (Wilcoxon rank test) was applied for testing differences between the study groups for quantitative parameters. All tests applied were two tailed, and $p \leq 5\%$ was considered statistically significant. Data were analyzed using SAS software (SAS Institute, Cary, NC, USA).

Results

Effect of continuous TEPA treatment on $CD34^+$ cell expansion

Purified cord blood-derived $CD34^+$ cells were cultured in cytokine-supplemented liquid medium in the *continuous* presence or absence of 5 μM TEPA. To measure more precisely the content of $CD34^+$ cells in the culture, $CD34^+$ cells were reselected from about half of the culture at weeks 3, 7, 9, and 11 and counted. Fold expansion was calculated as described in the Materials and methods section. The results showed an intrinsic variability in the ex vivo expansion potential of $CD34^+$ cells derived from different cord blood units. Nevertheless, the use of TEPA substantially increased the renewal potential, resulting in prolongation of $CD34^+$ cell expansion in the long-term cultures (weeks 7–11) (Table 1). During the first weeks of culture, $CD34^+$ cell expansion was similar in both the untreated (control) and TEPA-treated cultures.

Effect of short-term TEPA treatment on long-term cell expansion

In another set of experiments, ex vivo cultures were treated for 3 weeks only (treatment phase) with TEPA and cytokines (TEPA-treated) or with cytokines only (control). Then, from week 3 onward (up to 11 weeks), both cultures were treated with cytokines only (assay phase).

Determination of total nucleated cells (TNC), $CD34^+$ cells and CFUc at the termination of the treatment phase (week 3) demonstrated similar cumulative numbers in both TEPA-treated and control cultures. In contrast, at the termination of the assay phase (weeks 7–11), TEPA-pretreated cultures continued to sustain substantial proliferation of $CD34^+$ cells and CFU-C, whereas the control cultures lost most of these progenitor cells. In 8 of 10 experiments, the cumulative numbers of TNC, $CD34^+$ cells, and CFU-C at the end of the assay phase were higher in TEPA-pretreated cultures compared with that in the control cultures ($p = 0.01$, 0.01 and 0.002, respectively) (Table 2).

Table 1. Effect of TEPA treatment on long-term $CD34^+$ cell expansion

		Fold expansion of $CD34^+$ cells			
		3 Weeks	7 Weeks	9 Weeks	11 Weeks
Exp 1	Control	19	13	8	0
	TEPA	21	154	132	132
Exp 2	Control	20	22	132	88
	TEPA	25	189	1415	6012
Exp 3	Control	18	82	0	0
	TEPA	19	192	1415	1492
Exp 4	Control	7	0	0	0
	TEPA	10	86	131	246
Exp 5	Control	13	139	235	ND
	TEPA	11	288	683	ND
Exp 6	Control	9	25	0	0
	TEPA	20	49	44	88
Mean	Control	14 ± 2.24	46 ± 21	62 ± 40	17 ± 17
	TEPA	17 ± 2.41	159 ± 35	636 ± 263	1594 ± 1134
	p	0.2	0.016	0.05	0.012

Each of the six experiments was initiated with 1.2×10^5 $CD34^+$ cells derived from a single cord unit.

The cells were cultured with and without (control) 5 μM TEPA. At the indicated weeks, $CD34^+$ cells were repurified and counted. Fold expansion was calculated as described in the Materials and methods section.

p = significance of the difference between control and TEPA-treated cultures.

Analysis of cell morphology did not reveal any difference between the TEPA-treated and the control cultures at the end of the treatment phase. In the assay phase, TEPA-pretreated cultures contained mostly immature cells, whereas the control cultures contained mainly differentiated cells (Fig. 1 A and B).

The finding that short-term treatment resulted in long-term effect suggests that TEPA modulates either lineage-specific differentiation and/or the self-renewal potential of an early $CD34^+$ cell subset.

Effect of short-term TEPA treatment on early progenitors, lineage-committed progenitors and differentiated cells

$CD34^+$ hematopoietic progenitors comprise a heterogeneous population. The minority, $CD34^+CD38^-$ and $CD34^+Lin^-$ cells, are lineage uncommitted progenitors, whereas the majority, $CD38^+$ and Lin^+ cells, are lineage committed [13,14]. In the presence of cytokines (such as the combination used in the present study), the bulk of the $CD34^+$ cells undergoes myelomonocytic maturation and acquires surface antigens, including CD33, CD14, and CD15. At early stages of maturation, the cells coexpress CD34 and the myelomonocytic markers, whereas at later stages of differentiation CD34 is lost.

To determine the effect of TEPA on the various cell subsets, cultures were initiated with eight cord blood units either in the presence or absence of TEPA. After 3 weeks, we analyzed the percentage of cells expressing lineage-specific antigens: (myeloid [CD33, CD14, CD15], megakaryocytic [CD41, CD61], lymphoid [CD3, CD4, CD 19, CD56]), intermediate phenotype cells coexpressing CD34 and lineage-specific antigens, and cells expressing early phenotype antigens ($CD34^+CD38^-$ and $CD34^+Lin^-$).

The results indicated that the percentage of myeloid, $CD33^+$, and $CD15^+$ cells was similar in both the TEPA-treated and the control cultures. The percentage of cells expressing the monocytic CD14 antigen was 9 ± 1 in the TEPA cultures and 14.5 ± 2.4 in the control cultures (Fig. 2A). The percentage of committed myelomonocytic progenitor cells ($CD34^+CD33^+$, $CD34^+CD15^+$, $CD34^+CD14^+$) also was similar in the TEPA-treated and control cultures (Fig. 2B). We also compared the share of cells expressing lymphoid (CD19, CD56, CD3, CD4) and megakaryocytic (CD61, CD41) surface antigens in the total population (Fig. 2C) and in the $CD34^+$ cell population (Fig. 2D). Again, only minor differences were observed between the TEPA and the control cultures during the treatment phase.

Early and intermediate progenitors were measured after immunomagnetic bead purification of $CD34^+$ cells. The results indicated significantly higher percentages of $CD34^+CD38^-$ and $CD34^+Lin^-$ cells in TEPA-treated cultures (21.4 ± 4 and 6.8 ± 2, respectively), compared with those in the control cultures (2.7 ± 1 and 0.9 ± 0.3, respectively) (Fig. 3A). Representative FACS analysis of TEPA-treated and control cells with respect to CD34/CD38 and CD34/CD38/Lin is shown in Fig. 3B. Accordingly, the absolute content of $CD34^+CD38^-$ and $CD34^+CD38^-Lin^-$ cells was significantly higher in TEPA-treated ($67.6 \times 10^4 \pm 21$ and $22 \times 10^4 \pm 7.7$ respectively) than in control cultures ($7.4 \times 10^4 \pm 3$ and $2.7 \times 10^4 \pm 1.2$ respectively).

Table 2. Effect of short-term treatment with TEPA on short- and long-term cultures

	CD34 cells $\times 10^5$				CFU-C $\times 10^4$				TNC $\times 10^6$			
	Short term		Long term		Short term		Long term		Short term		Long term	
Exp. No.	Control	TEPA	Control	TEPA	Control	TEPA	Control	TEPA	Control	TEPA	Control	TEPA
1	50	79	219	1404	278	233	215	1475	73	72	1843	6144
2	59	50	374	1584	252	264	409	2621	72	54	668	2304
3	51	25	0	1075	ND	ND	ND	ND	65	10	92	937
4	9	13	131	197	26	48	0	655	25	35	245	860
5	9	5	51	51	29	25	614	123	25	20	983	645
6	37	20	0	92	346	173	0	737	60	36	31	307
7	28	15	0	1536	254	315	0	1982	41	33	246	1751
8	16	29	5	61	ND	ND	3	375	32	48	100	491
9	9	11	0	42	142	230	0	543	58	50	284	806
10	12	16	49	921	204	269	62	3225	91	77	368	7865
Mean ± SE	28 ± 6	26 ± 7	82 ± 39	696 ± 212	191 ± 41	194 ± 37	144 ± 75	1304 ± 363	54 ± 7	43 ± 6	486 ± 176	2211 ± 830
p		0.9		0.01		1.0		0.002		0.3		0.01

Each experiment was initiated with 1.2×10^5 $CD34^+$ cells derived from a single cord unit and cultured according to a two-phase procedure: a 3-week treatment phase, during which the cultures were supplemented with both TEPA and cytokines, and an assay phase, from week 3 onward, during which TEPA was removed and the cultures were supplemented with cytokines alone. Control cultures were treated with cytokines only in both phases. $CD34^+$ cells, CFU-C, and total nuclear cells (TNC) were determined after 3 weeks (short term) and after 7–11 weeks (long term). Cumulative numbers are shown and calculated as described in the Materials and methods section.

p = significance of the difference between control and TEPA-treated cultures.

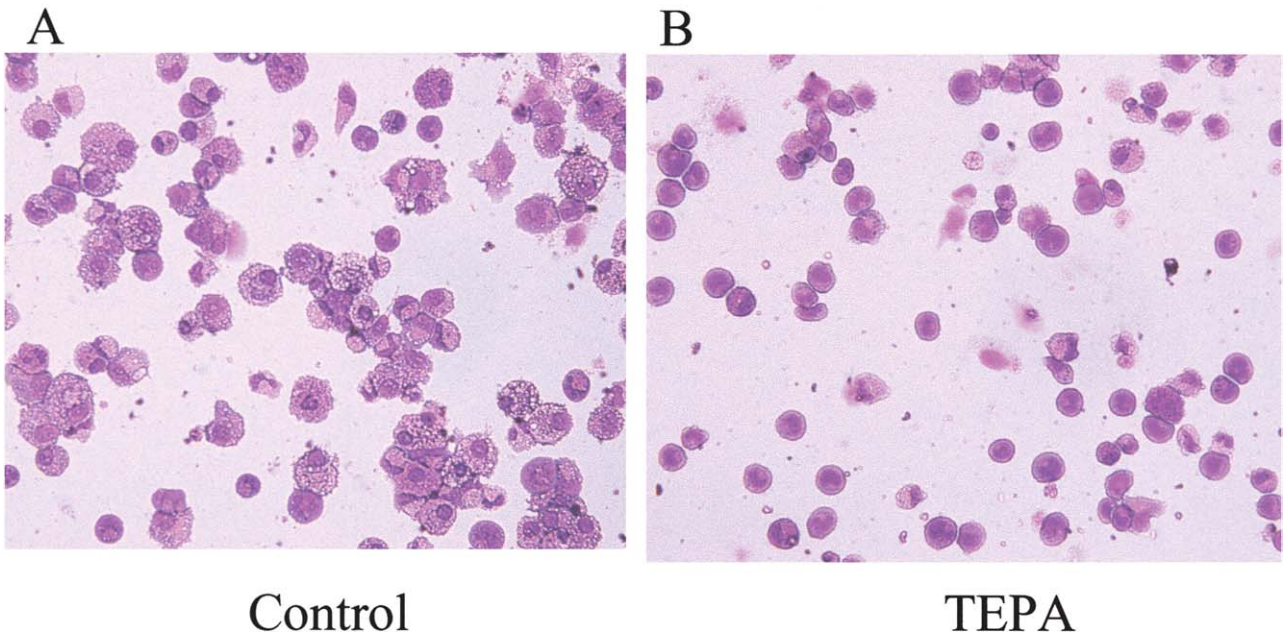

Figure 1. Cell morphology of TEPA-treated and untreated cultures. Photomicrographs of Giemsa-stained cells derived from 8-week cultures grown in the (**A**) absence (control) or (**B**) presence of TEPA (5 μM) for the first 3 weeks of culture (magnification ×400).

The content of $CD34^+$ cells was still similar in both treated ($311 \times 10^4 \pm 68$) and untreated ($298 \times 10^4 \pm 56$) cultures (Table 3).

TEPA enhances marrow-repopulating ability in NOD/SCID mice

A quantity of 0.5×10^4 $CD34^+$ freshly purified noncultured cells or 30×10^4 of their progeny $CD34^+$ cells repurified from 5-week TEPA-treated or control cultures was transplanted into sublethally irradiated NOD/SCID mice. Fold expansion of $CD34^+$ cells in both cultures was $60\times$ that of the input. In addition, all experimental groups were cotransplanted with 2×10^6 irradiated supportive cells taken from the $CD34^-$ fraction of the noncultured cells. The percentage of human $CD45^+$-expressing cells, human $CD45^+$-expressing progenitor cells ($CD45^+CD34^+$), and lineage differentiated cells in the mouse BM was evaluated 8 weeks after transplantation.

We found that injection of TEPA-treated $CD34^+$ cells resulted in $13.1 \pm 5\%$ ($n = 7$) engrafted $CD45^+$ cells in the mouse BM. The engraftment potential of $CD34^+$ cells derived from TEPA-treated cultures was significantly greater than that of $CD34^+$ cells derived from TEPA-untreated (control) cultures ($0.73 \pm 0.36\%$, $p = 0.03$) and noncultured $CD34^+$ cells ($0.59 \pm 0.35\%$, $p = 0.02$) (Fig. 4A). Irradiated mice transplanted with supportive cells only ($CD34^-$ irradiated cells) demonstrated 0.07 to 0.12% $CD45^+$ cells. FACS analysis of the human progenitor cells $CD45^+CD34^+$ showed superiority of TEPA-treated cells over that of TEPA-untreated and noncultured cells (Fig. 4B). Fig. 4C shows that TEPA-treated cells sustain the potential to differentiate into myeloid (CD33, CD14, CD15), lymphoid (CD19), megakaryocytic (CD61, CD41), and erythroid (GPA) lineages following engraftment in SCID mice.

Cumulatively, our data suggest that during the first weeks of culture, TEPA specifically increases the expansion of the early progenitor cell subsets $CD34^+CD38^-$ and $CD34^+Lin^-$ but has no effect on the total number of $CD34^+$ cells. This preferential expansion most likely is the driving force behind the longer duration of $CD34^+$ cell expansion in vitro and the higher in vivo SCID engraftment potential.

Discussion

Different combinations of cytokines support extensive proliferation of human stem and progenitor cells that usually is accompanied by commitment and differentiation [15–18]. Early-acting cytokines, such as SCF, TPO, Flt3-ligand, and IL6, support prolonged proliferation compared with mixtures of late-acting cytokines, e.g., IL3, G-CSF, and erythropoietin, which accelerate differentiation and reduce the duration of active proliferation. Nonetheless, conditions that modulate the balance between self-renewal and differentiation of hematopoietic stem and progenitor cells as imposed by different cytokine combinations are not well defined. We recently reported that modulation of cellular Cu content might shift the balance between self-renewal and differentiation, resulting in acceleration or delay of cell differentiation [11].

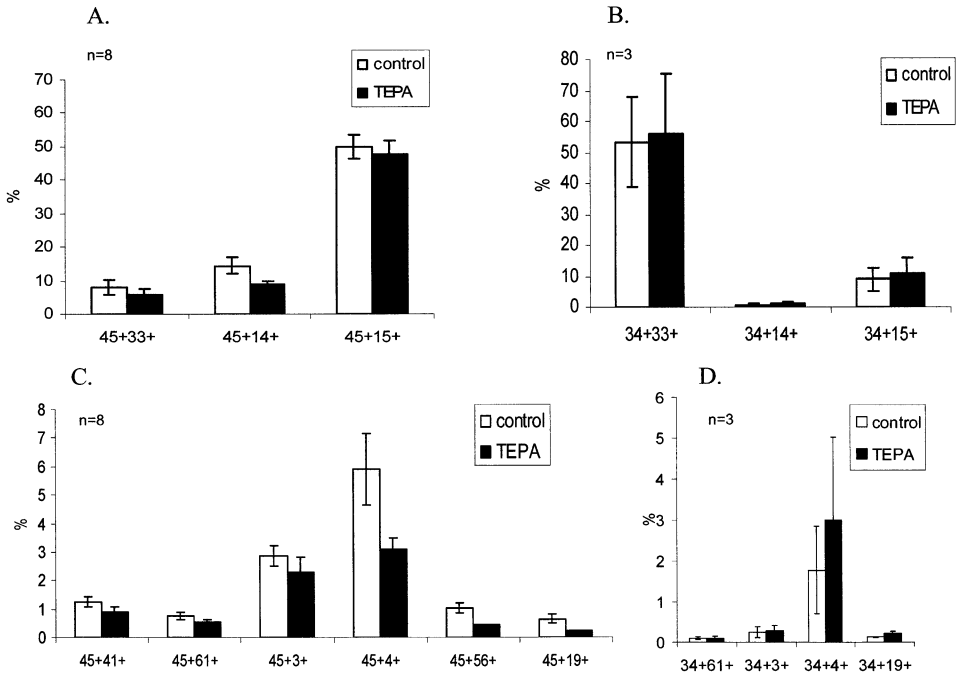

Figure 2. FACS analysis of lineage-committed progenitors and differentiated cells. Each experiment was initiated with cells derived from single cord units. The percentages of myeloid (CD14, CD15, CD33) (**A**), lymphoid (CD3, CD4, CD19, CD56), and megakaryocytic (CD41, CD61) (**C**) cells were determined after 3 weeks of growth in the absence (control) or presence of TEPA. In parallel, $CD34^+$ cells were repurified and stained for CD34 in combination with lineage-specific antigens. The percentages of myeloid ($CD34^+CD15^+$, $CD34^+CD14^+$, $CD34^+CD33^+$) (**B**) and lymphoid ($CD34^+CD3^+$, $CD34^+CD4^+$, $CD34^+CD19^+$) and megakaryocytic ($CD34^+CD61^+$) (**D**) committed progenitor cells are shown.

Cu has a marked effect in various biologic systems. Cu-specific chelators blocked aerial hyphae formation in *Streptomyces,* illustrating the crucial role of Cu in morphogenesis. On the other hand, elevated Cu concentrations in the growth medium bypassed the developmental block of ram-dependent *Streptomyces lividans* differentiation, suggesting that high Cu levels rendered the ram-associated factor obsolete [19]. An increase in the extracellular concentration of Cu modulated the ability of mesenchymal stem cells to differentiate into osteoblasts or adipocytes [20]. In the hematopoietic system, Cu is essential to the proliferation and differentiation of BM-derived progenitor cells [8,9].

In the present study, we demonstrated that the low-molecular-weight linear polyamine Cu chelator TEPA at a concentration that moderately reduced cell Cu content (by 20–30%) enabled extensive *ex vivo* expansion of $CD34^+$ cells in cultures supplemented with early-acting cytokines (SCF, TPO, Flt3-ligand, IL6). Deterioration of the control cultures occurred at a time when the TEPA-treated cultures continued to expand. As a result, at weeks 9 to 11, expansion of $CD34^+$ cells was by 1 to 3 logs higher in the TEPA-treated cultures compared with that in the controls (Table 1). A similar phenomenon was observed in cultures supported by combinations of late-acting cytokines. In this case, the optimal TEPA concentration was higher (10 μM) than in cultures treated with early-acting cytokines (data not shown). The effect of other linear polyamine Cu chelators, such as tetraethylentetramine and pentaethylenhexamine, on the ex vivo expansion of $CD34^+$ cells was similar to that of TEPA (manuscript in preparation).

In an attempt to elucidate the mechanism of the TEPA effect, we adopted a two-phase culture procedure: an initial 3-week treatment phase, during which the cultures were supplemented with both TEPA and cytokines, and an assay phase, from week 3 onward, during which TEPA was removed and the cultures were supplemented with cytokines alone. The results indicate that long-term CFU-C and $CD34^+$ cell expansion in the assay phase persists even in cultures supplemented with TEPA during the first 3-week treatment phase only (Table 2). These findings suggest inhibition of lineage-specific differentiation or modulation of the self-renewal potential of a $CD34^+$ cell subset. Determination of the culture composition during the treatment phase did not reveal any significant difference in the cumulative number of TNC, $CD34^+$ cells and CFUc (Table 2) or in the percentage of myelomonocytic lineage-specific committed progenitor cells (Fig. 2B). In contrast, higher percentages of early progenitors ($CD34^+38^-$, $CD34^+CD38^-Lin^-$) were found in TEPA-treated cultures compared with that in control cultures (Fig. 3A).

It has been reported that in vivo, such cells, although a minority (<10%) among the $CD34^+$ cells, are the most

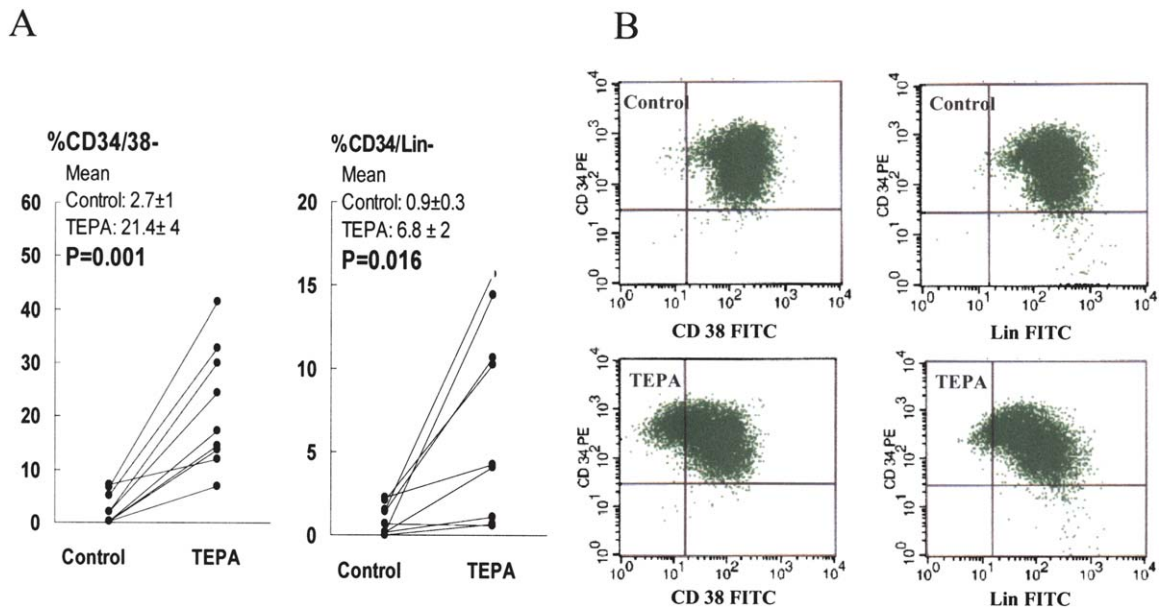

Figure 3. FACS analysis of $CD34^+$ early subsets. $CD34^+$ cells were repurified from 3-week control and TEPA-treated cultures using a MiniMACS CD34 progenitor cell isolation kit. The purified cells were stained for CD34/CD38 and CD34/Lin (CD38, CD33, CD14, CD15, CD3, CD61, CD19). The percentages of $CD34^+CD38^-$ and $CD34^+Lin^-$ cells are shown in (**A**); representative FACS analysis dot plot of $CD34^+$ cell subsets is shown in (**B**).

immature progenitor cells, which are capable of self-renewal and multilineage differentiation. The $CD34^+CD38^-$ cell fraction contains more long-term culture initiating cells (LTC-IC) pre-CFU [21] and exhibits longer maintenance of their phenotype [22]. $CD34^+CD38^-$ cells can give rise to lymphoid and myeloid progeny in vitro [14] and have an enhanced capacity to repopulate SCID mice [23,24]. Moreover, in patients who received autologous stem cell transplantation, the number of $CD34^+CD38^-$ cells infused correlated positively with the speed of hematopoietic recovery [25,26].

To compare the SCID engraftment potential of cells cultured under different protocols, TEPA-treated and TEPA-untreated cultures were harvested at week 5. At this time, no difference in $CD34^+$ cell expansion was discerned between the cultures. The $CD34^+$ cells were repurified using a MiniMACS isolation kit and transplanted into sublethally irradiated NOD/SCID mice. A quantity of 30×10^4 purified cells, the yield of cultures initiated with 0.5×10^4 cord blood-derived $CD34^+$ cells, either from TEPA-treated or TEPA-untreated cultures, was injected into each mouse.

The results showed that although a similar number of $CD34^+$ cells was transplanted from both cultured groups,

Table 3. Analysis of three-week cultures

	Cells × 10^4							
	TNC		$CD34^+$		$CD34^+$ $CD38^-$		$CD34^+Lin^-$	
Exp. No.	Control	TEPA	Control	TEPA	Control	TEPA	Control	TEPA
1	6096	3264	126	123	8	51	3	5
2	3552	2784	237	165	17	20	2	1
3	6528	4656	142	263	0.3	18	0	2
4	6144	7968	466	655	1.4	113	0.6	27
5	10608	7056	221	259	4.5	78	3	41
6	8640	6624	205	155	4.4	38	3	17
7	5088	4176	204	261	0.6	38	0.3	3
8	4032	1440	524	252	1.3	35	1	36
9	7440	6480	556	666	29	219	11	68
Mean (±SE)	6459 ± 740	4939 ± 738	298 ± 56	311 ± 68	7.4 ± 3.2	67.6 ± 21	2.7 ± 1.2	22.1 ± 7.7
p		0.6		0.8		0.0005		0.009

Each experiment was initiated with 1.2×10^5 $CD34^+$ cells derived from a single cord unit. The absolute number of total nuclear cells (TNC), $CD34^+$, $CD34^+CD38^-$ and $CD34^+Lin^-$ cells was calculated ad described in the Materials and methods section.

p = significance of the difference between control and TEPA-treated cultures.

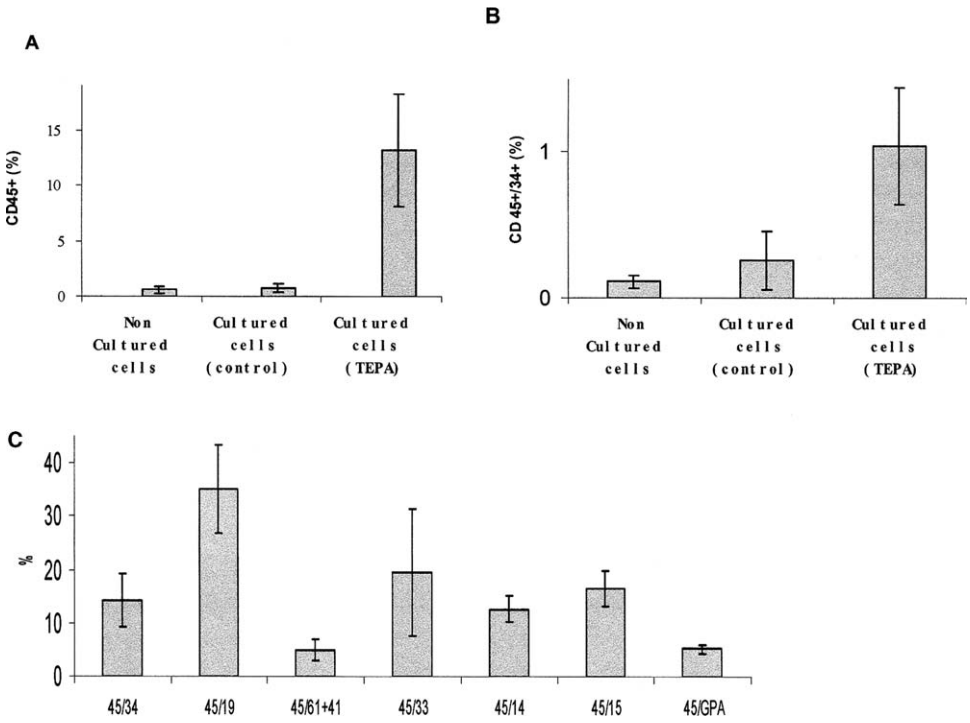

Figure 4. NOD/SCID engraftment potential of TEPA-treated and TEPA-untreated cultured cells. A quantity of 0.5×10^4 noncultured $CD34^+$ cells or the yield from TEPA-treated and TEPA-untreated (control) cultures initiated with 0.5×10^4 $CD34^+$ cells were transplanted into the experimental mice, as described in the Materials and methods section. Cultured cells were harvested after 5 weeks and $CD34^+$ cells were repurified. In both TEPA-treated and control cultures, similar numbers of $CD34^+$ cells were obtained; 30×10^4 $CD34^+$ cells were injected per mouse together with 2×10^6 irradiated supportive cells taken from the $CD34^-$ fraction of the noncultured cells. Negative control mice received only irradiated $CD34^-$ cells. At 8 weeks, the mice were sacrificed and the BM harvested from both femurs and tibiae. Human cell engraftment was assessed by the number of cells positively stained by anti-human CD45 antibodies (**A**) and human progenitor cells by the number of cells positively stained by both anti-human CD45 and anti-CD34 (**B**). The mean percentages (±SE) of human cells in the mouse BM are shown. FACS analysis of cells double stained with PE anti-CD45 and FITC anti-lineage specific antigens evaluated lineage-specific differentiation potential of TEPA-treated cells. Data are shown as percentages of $CD45^+$ cells (total human cell engraftment) (**C**).

cells derived from TEPA-treated cultures have a significantly higher SCID engraftment potential than cells derived from the control, TEPA-untreated cultures ($p = 0.03$) (Fig. 4A). Although the engraftment potential of control cultured cells was comparable to the respective fraction of cells before culture, the engraftment of TEPA-treated cultured cells was significantly higher ($p = 0.02$).

Taken together, our results suggest that during the first few weeks of ex vivo expanded cell growth, TEPA enables the preferential proliferation of a subset of $CD34^+$ cells with a $CD34^+CD38^-$ and $CD34^+Lin^-$ immunophenotype that have an increased in vivo and ex vivo self-renewal potential. Expansion under ex vivo conditions of early $CD34^+$ progenitor cell subsets may have a clinical application for cord blood transplantation.

Acknowledgments

This work was presented in part at the 9th meeting of the International Society of Cell Therapy (Phoenix, Arizona, USA, May 2003) and at the 32nd annual meeting of the International Society of Experimental Hematology (Paris, France, July 2003).

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