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Chelatable cellular copper modulates differentiation and self-renewal of cord blood–derived hematopoietic progenitor cells

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Objectives. **We have demonstrated epigenetic modulation of $CD34^+$ cell differentiation by the high-affinity copper (Cu) chelator tetraethylenepentamine (TEPA). TEPA slowed down the rate of $CD34^+$ cell differentiation and increased their engraftability in SCID mice. TEPA biological activity was attributed to its effect on cellular Cu levels as (a) treatment with TEPA resulted in reduction of cellular Cu, and (b) excess of Cu reversed TEPA's activity and accelerated differentiation. In the present study we further evaluated the role of cellular Cu in TEPA's biological activity.**

Methods. **The effects of Cu-chloride, TEPA, TEPA/Cu mixtures at various ratios, and a synthesized, stable, TEPA-Cu complex on short- and long-term cord blood–derived $CD34^+$ cell cultures as well as on the overall and chelatable cellular Cu were investigated.**

Results. **Addition of TEPA, TEPA/Cu mixtures at up to equimolar concentrations, and the TEPA-Cu complex to $CD34^+$ cell cultures resulted in inhibition of differentiation and enhancement of long-term self-renewal. Measurement of the overall cellular Cu by atomic absorption spectrophotometry showed 20 to 40% decrease by TEPA while the TEPA-Cu mixture and the TEPA-Cu complex increased cellular Cu by 10- to 20-fold, as did $CuCl_2$. However, measurement of the cellular pool of labile Cu showed similar reduction (50% from the control) by all the TEPA forms, while $CuCl_2$ increased it. Thus, inhibition of differentiation and enhancement of self-renewal of $CD34^+$ cells was correlated with reduction in the cellular chelatable Cu content.**

Conclusion. **The results suggest that decreasing of the chelatable Cu pool, rather than overall Cu, is the mechanism that stands behind TEPA's biological activity.** © **2005 International Society for Experimental Hematology. Published by Elsevier Inc.**

Introduction

Metal ions such as iron (Fe), calcium (Ca), magnesium (Mg), and zinc (Zn) are known to play important roles in basic cell functions such as cell survival, proliferation, and differentiation. Relatively little attention, however, has been drawn to the role of copper (Cu) in key cellular functions, despite well-documented and significant clinical manifestations of Cu deficiency [1–3]. The symptoms of such deficiency involve several organ systems, yet of particular relevance to this study is the fact that Cu deficiency is often associated with hematopoietic cell differentiation arrest, which results in anemia, neutropenia, and thrombocytopenia [1–3]. These pathological manifestations are unresponsive to iron therapy but are rapidly reversed following Cu supplementation [1–5]. Morphological and functional evaluation of the bone marrow (BM) of neutropenic, Cu-deficient patients demonstrates the striking absence of mature cells (''maturation arrest'') along with the presence of intact progenitor cells. This finding suggests that the shortage of functional circulating blood cells in these patients is due to a block in development of the hematopoietic stem/progenitor cells (HSPCs) in a Cu-deficient microenvironment [1].

Further insight into the role of Cu in hematopoiesis comes from studies with established cell lines. Bae and Percival [6] have demonstrated that retinoic acid–induced

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HL-60 cell differentiation was associated with accelerated uptake of Cu during the early stages of differentiation. Accordingly, addition of excess Cu to the culture medium sensitized the cells to retinoic acid–induced differentiation [7]. Ceruloplasmin, the main Cu-binding protein in serum, was demonstrated in vitro [8] and in vivo [9] to be a potent inducer of hematopoietic cell differentiation. In this context, it is interesting to note that ceruloplasmin has been shown to have a therapeutic effect in patients with aplastic anemia [9]. While excess Cu was associated with enhanced differentiation, Cu-deficient cells displayed suboptimal responses to several differentiation signals; reduction of Cu in U937 cells by the polyamine Cu-chelator triethylenetetramine was shown to inhibit cell differentiation induced by 1,25-dihydroxyvitamin D3 and phorbol 12-myristate 13-acetate [10].

To gain insight into the role of Cu in the regulation of HSPC proliferation and differentiation, we used cultures of cord blood (CB)-derived purified $CD34^+$ cells grown in cytokine-supplemented liquid medium. Cellular Cu concentration was moderately modulated by addition of Cu or a Cu chelator, tetraethylenepentamine (TEPA) [11]. Treatment with TEPA resulted in enrichment of progenitor subsets ($CD34^+CD38^-$ and $CD34^+CD38^-Lin^-$) that displayed prolonged ex vivo expansion of CFUc and $CD34^+$ cells and an enhanced capacity to repopulate NOD/SCID mice [12,13]. In contrast, treatment with Cu chloride resulted in a marked decrease in $CD34^+$ cells and the early subsets and, consequently, in their long-term culture potential. These results suggested that changes in the cellular Cu mediated the biological effects of these reagents. Indeed, we demonstrated that only Cu, but not other transitional metal ions, could reverse TEPA's effect [11]. However, this reversal was achieved only with excess of Cu. At equimolar ratio, Cu did not quench TEPA's effect.

In the present study, we reevaluate the role of Cu in HSPC self-renewal and differentiation. For this purpose, we synthesized a stable TEPA-Cu complex and compared its effect on CD34 cells to that of the TEPA:Cu (1:1) mixture and TEPA. The results indicated similar biological activity for all these reagents. Yet, measurement of the overall cellular Cu content indicated that while TEPA decreased it, the TEPA:Cu (1:1) mixture and the complex, as well as Cu chloride, which has an opposite biological activity, decreased it.

Cellular Cu is mostly bound to various cellular components such as ceruloplasmin and various enzymes such as Cu/Zn superoxide dismutase. Very little exists as loosely bound, labile ions. The labile form of Cu can be quantified by its ability to bind to cell-permeable chelators, and thus it is operationally characterized as chelatable Cu. We determined the chelatable Cu pool by its effect on the fluorescence of the cell-permeable chelator calcein acetoxymethyl ester as measured by flow cytometry. The results indicated that TEPA in all its forms decreased this Cu pool, while Cu chloride increased it. These results suggest that reduction in the chelatable Cu pool rather than that of the overall Cu content is the mechanism that stands behind the effect of TEPA on cord blood–derived $CD34^+$ cells.

Materials and methods

Purification of cord blood–derived CD34 cells

Cells were separated from umbilical cord blood obtained from normal full-term deliveries from Chaim Sheba Medical Center, Tel Hashomer, Israel (informed consent was given). Samples were collected and frozen according to Rubinstein et al. [14] within 24 hours postpartum. Prior to use, the cells were thawed, and $CD34^+$ cells purified by immunomagnetic bead separation using a Mini-MACS CD34 progenitor cell isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany), according to the manufacturer's recommendations.

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, interleukin-6, FLT-3 ligand, stem cell factor (each at a final concentration of 50 ng/mL), and interleukin-3 at 20 ng/mL (Pepro Tech, Inc., Rocky Hill, NJ, USA), and incubated at 37°C in a humidified atmosphere of 5% CO_2 in air. The cultures were topped weekly with the same volume of fresh medium up to week 3, and then up to the termination of the experiment the cultures were weekly demi-depopulated.

The two-phase culture assay

To evaluate the biological effect of various forms of tetraethylenepentamine (TEPA) and Cu chloride (Aldrich, Milwaukee, WI, USA), cultures were treated for 3 weeks (treatment phase) with a specific reagent or combination of these reagents, as indicted, in addition to cytokines, while control cultures were treated with cytokines only. From week 3 on, both experimental and control cultures were treated with cytokines only for an additional 5 weeks (assay phase). CFUc and $CD34^+$ cells were assayed as previously described [12] to determine the effect of specific treatment on the long-term culture potential.

Immunostaining and flow cytometry

The cells were washed with a phosphate-buffered saline (PBS) solution containing 1% bovine serum albumin (BSA), and stained (at 4°C for 30 minutes) with fluorescein isothiocyanate (FITC)- or phycoerythrin (PE)-conjugated antibodies. The cells were then washed in the above 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 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

$CD34^+$ cells were measured in a purified, reselected fraction, using the MiniMACS CD34 progenitor cell isolation kit as described above (purification of cord blood–derived CD34 cells). $CD34^+$ cell content of the entire culture was calculated as follows: Number of $CD34^+$ cells recovered following repurification $\times$ 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 on, the culture volume was calculated by multiplying the actual volume by the number of passages. Fold expansion was calculated by dividing the $CD34^+$ cell content of the culture by the number of inoculated $CD34^+$ cells.

Determination of early $CD34^+$ cell subsets

The percentages of the early $CD34^+$ cell subsets were determined from the repurified $CD34^+$ cell fraction. Cells were washed and immuno-stained as described above with FITC anti-CD38 and PE anti-CD34 antibodies for determination of $CD34^+CD38^-$ cells and FITC anti-CD34 and PE anti–lineage-specific antibodies (anti CD38, CD33, CD14, CD15, CD3, CD61, CD19) (Becton-Dickinson) for determination of $CD34^+CD38^-Lin^-$ cells. Results are given as percentage of $CD34^+$ cells. Absolute numbers of $CD34^+CD38^-$ and $CD34^+CD38^-Lin^-$ cells in the culture were calculated from the total number of $CD34^+$ cells recovered following the repurification step.

Preparation of the TEPA-Cu complex

TEPA·5 HCl (3 mmol, 1.1 g, obtained from Sigma) was treated with a 15-mL solution of 1 N NaOH in methanol. The precipitate of NaCl was separated by centrifugation at 3000 rpm for 5 minutes. The solution of TEPA base was diluted with 120 mL methanol and a light blue 30-mL solution of 3 mM $CuCl_2$ in H_2O was added. A bright blue color solution was formed. The reaction solution was evaporated under vacuum at 25 to 30°C. The residue was diluted with 100 mL methanol and again evaporated under vacuum to remove water. This process was repeated twice. The residue was dissolved in isopropanol (15 mL) and the resulting NaCl precipitate was removed by filtration. The filtrate solution was diluted with diethyl ether (45 mL) and the resulting solution was recrystallized at 8 to 10°C for 2 weeks to obtain the crystallized TEPA-Cu complex. The solution was filtered out, and the resulting recrystallized solid material (dark blue precipitate) on the walls of the flask was washed with diethyl ether (50 mL). The ether was removed and the solid product was dried under vacuum, yielding 0.74 g of dark blue solid TEPA-Cu complex product. No traces of residual free Cu or TEPA were detected by fast atom bombardment mass spectrometry (FAB-MS) [15].

Cu determination

Overall cellular Cu content was determined as previously described [7]. In brief, cells were harvested and washed with PBS. Aliquots of 2×10^6 cells in metal-free Eppendorf tubes were pelleted and dissolved with 0.03 mol/L ultra-pure nitric acid (Mallinckrodt Baker B.V., Deventer, Holland). Samples were sonicated and then analyzed by graphite furnace atomic absorption spectrophotometry using a model 460 spectrophotometer with a HGA 2200 controller (Perkin Elmer, Norwalk, CT, USA).

Cellular chelatable Cu was measured as follows: cells were washed twice with saline and incubated at a density of 0.5 to 1 $\times$ 10^6/mL with 0.25 mM calcein acetoxymethyl ester (CA-AM) for 15 minutes at 37°C. Then, the cells were washed twice and exposed to either TEPA, the TEPA:Cu (1:1) mixture, the TEPA-Cu complex, or none, as indicated. Cellular fluorescence was measured after incubation with CA-AM and 3 hours thereafter by flow cytometry using a 488-nm argon laser for excitation and the FL1 PMT for measuring emission. Unstained cells served to determine background fluorescence. CellQuest software (Becton-Dickinson) was used to calculate the mean fluorescence channel of the studied cell population in arbitrary fluorescence units.

The procedure is based on the ability of CA-AM to enter viable cells and to become fluorescent upon hydrolysis [16,17]. Following binding of Cu calcein fluorescence is quenched. This quenching is much greater than that caused by iron or any other metal ion [18,19]. The decrease in fluorescence under different conditions measures, in relative terms, the chelatable Cu pool.

Statistics

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 value of 5% or less was considered statistically significant. The data were analyzed using SAS software (SAS Institute, Cary, NC, USA).

Results

The effect of Cu on $CD34^+$ cell cultures

CB-derived $CD34^+$ cells were treated with cytokines and 10 μM Cu chloride during the first 3 weeks of the culture (treatment phase) and then with cytokines only for additional 5 weeks (assay phase). Analysis of the cultures at the end of the treatment phase indicated that the number of total nuclear cells (TNC) was similar in cultures treated or untreated with Cu (Fig. 1A). The CFUc content of the Cu-treated cultures was 1.6-fold lower than in the untreated cultures (170 ± 78 vs 272 ± 172, respectively, $n = 3$), but this difference did not reach statistical significance ($p > 0.05$) (Fig. 1B). At the end of the assay phase (week 8), significantly lower CFUc were found in Cu-treated cultures than in control cultures ($43 \pm 11 \times 10^3$ vs $124 \pm 9 \times 10^3$, respectively, $n = 3$, $p < 0.05$ (Fig. 1C). FACS analysis of the subset cell composition on week 3 of cultures treated with different concentrations of Cu (5–20 μM) revealed remarkably lower absolute numbers of $CD34^+$, $CD34^+CD38^-$, and $CD34^+CD38^-Lin^-$ cells in Cu-treated cultures (Fig. 2A–C). Notably, at all the tested concentrations, Cu chloride treatment did not adversely affect the TNC number during the treatment phase (Fig. 2D), suggesting that the Cu treatment specifically impaired the proliferation of progenitor cells.

The effect of simultaneous treatment with Cu and TEPA on $CD34^+$ cell cultures

Next, we tested the effect of simultaneous treatment with TEPA and Cu on CFUc content (Fig. 3) and cell immunophenotype (Fig. 4). At up to equimolar concentration, Cu did not attenuate TEPA's effect on long-term

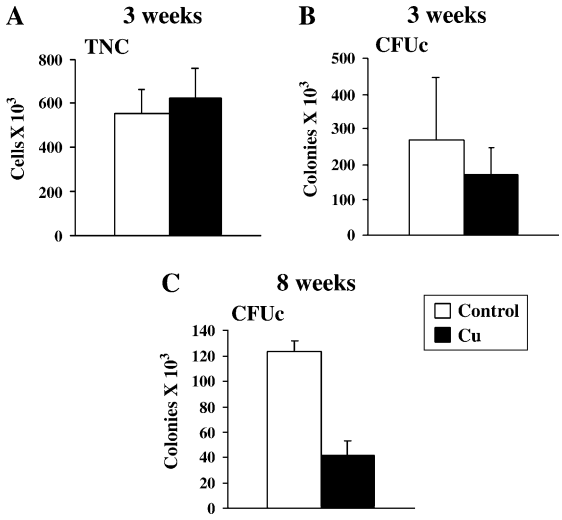

Figure 1. The effect of Cu on CD34$^+$ cell cultures. CD34$^+$ cell cultures (n = 3), grown in the presence of cytokines, were treated for 3 weeks with or without 10 μM Cu chloride. The numbers of TNC **(A)** and CFUc **(B)** were determined. From week 3 on, the cultures were grown with cytokines alone. CFUc content was determined on week 8 **(C)**. Cumulative numbers are shown.

(8-week) CFUc. Surprisingly, even at equimolar ratio (TEPA:Cu 1:1) the CFUc content of the cultures was comparable to that of only-TEPA-treated cultures and was significantly above that of control (nontreated) cultures. Only excess Cu (TEPA:Cu 1:2) overrode TEPA's effect

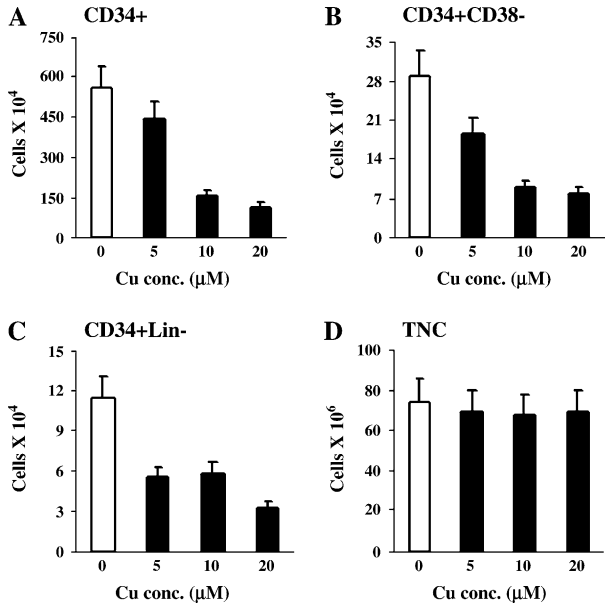

Figure 2. Analysis of the cell composition of 3-week cultures treated with Cu. CD34$^+$ cell cultures (n = 3), grown in the presence of cytokines, were treated for 3 weeks with or without different concentrations of Cu chloride. Absolute numbers of CD34$^+$ **(A)**, CD34$^+$CD38$^-$ **(B)**, and CD34$^+$CD38$^-$Lin$^-$ cells **(C)**, stained, analyzed, and calculated as described in Materials and methods, are demonstrated. TNC numbers are shown in **D**.

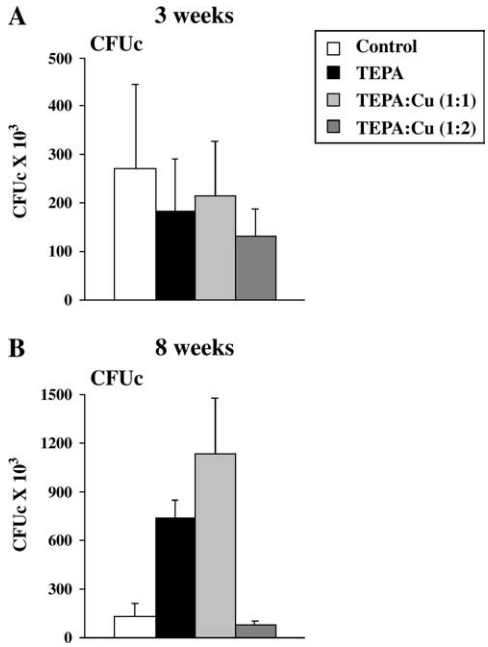

Figure 3. Effect of simultaneous treatment with TEPA and Cu at 1:1 and 1:2 molar ratios on short- and long-term CFUc expansion. CD34$^+$ cell cultures (n = 3), grown in the presence of cytokines with 10 μM TEPA, 10 μM TEPA + 10 μM Cu chloride (1:1), 10 μM TEPA + 20 μM Cu chloride (1:2), or none. From week 3 on, the cultures were grown with cytokines alone. CFUc were determined on weeks 3 **(A)** and 8 **(B)**. Cumulative numbers were calculated as described in Materials and methods.

(Fig. 3B). Notably, on week 3, the CFUc (Fig. 3A) and CD34$^+$ cell content (Fig. 4C) were comparable in control and treated cultures. In sharp contrast, at this time CD34$^+$ early cell subsets (CD34$^+$CD38$^-$ and CD34$^+$Lin$^-$) were higher in cultures treated with either TEPA:Cu 1:1 or TEPA alone compared to control cultures (Fig. 4A,B).

The effect of a TEPA-Cu complex on CD34$^+$ cell cultures

In order to further explore the effects of TEPA and Cu, we synthesized a stable TEPA-Cu complex. Mass-spectra analysis of the TEPA-Cu-complex in solution (Fig. 5A) indicated the presence of two peaks of TEPA-Cu complex with molecular mass of 252 and 287, which correspond to TEPA-Cu complex and TEPA–Cu chloride complex, respectively. The two-dimensional chemical structure of the ionized form is shown in Figure 5B. Peaks of free Cu (MW=63) and free TEPA (MW=190) as well as other analogs of the TEPA-Cu complex were not detected. A similar pattern was observed after one month incubation at 37°C, indicating the stability of the synthesized compound.

To evaluate its biological activity, CD34$^+$ cell cultures were treated for 3 weeks with cytokines in the absence or presence of various concentrations (15–40 μM) of the TEPA-Cu complex. Analysis of cultures on week 8 demonstrated a dose-related increase in CFUc in cultures pretreated with the TEPA-Cu complex at times when control cultures declined (Fig. 6A). The CD34$^+$ cell

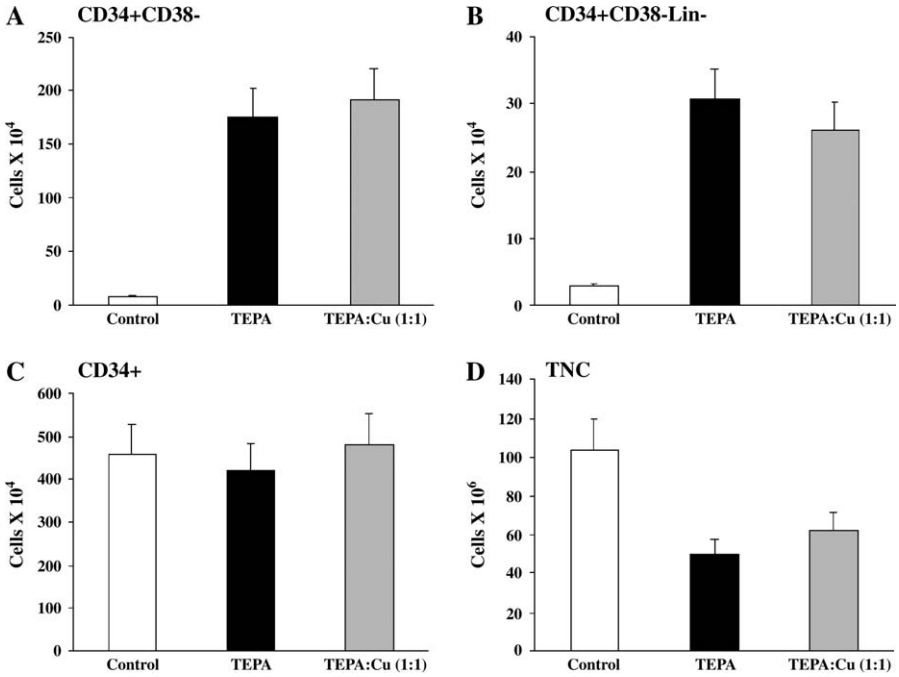

Figure 4. Effect of simultaneous treatment with Cu and TEPA on short-term expansion of $CD34^+$ cells. $CD34^+$ cells were treated for 3 weeks with 10 μM TEPA, 10 μM TEPA + 10 μM Cu chloride (1:1), or cytokines alone (n = 3). For FACS analysis of early progenitor subsets, $CD34^+CD38^-$ **(A)** and $CD34^+CD38^-Lin^-$ **(B)**, purified $CD34^+$ cells were stained, analyzed, and cumulative numbers were calculated as described in Materials and methods. Numbers of purified $CD34^+$ cells and TNC numbers are shown in **C** and **D**, respectively.

content of 8-week cultures treated with optimal concentrations of TEPA-Cu complex (40 μM) and TEPA (10 μM) were comparable ($27 \pm 10 \times 10^6$ and $27 \pm 9 \times 10^6$, respectively), and significantly above that of control cultures ($4 \pm 2 \times 10^6$, n = 4, $p < 0.05$) (Fig. 6B).

Phenotype analysis of 3-week cultures (Fig. 7A–C) demonstrated that the fold expansion of $CD34^+CD38^-$ cells in the treated cultures was significantly ($p < 0.05$) above that of control cultures (169 ± 40 and 21 ± 6, respectively). Similar results were obtained with $CD34^+CD38^-Lin^-$ cells (153 ± 26 and 37 ± 14, respectively), while $CD34^+$ cell expansion remained comparable to control cultures. To determine the specificity of the TEPA-Cu complex for TEPA's biological effects, we prepared and analyzed a TEPA-Zn complex. The preparation of this molecule was similar to that of the TEPA-Cu complex. Its biological analysis indicated no effect on CD34 cells (data not shown).

The effect of TEPA, TEPA:Cu (1:1), TEPA-Cu complex, and Cu chloride on cellular Cu content

Determination of the total cellular Cu content after treatment with TEPA, Cu-chloride, and the TEPA-Cu complex at various concentrations was performed by

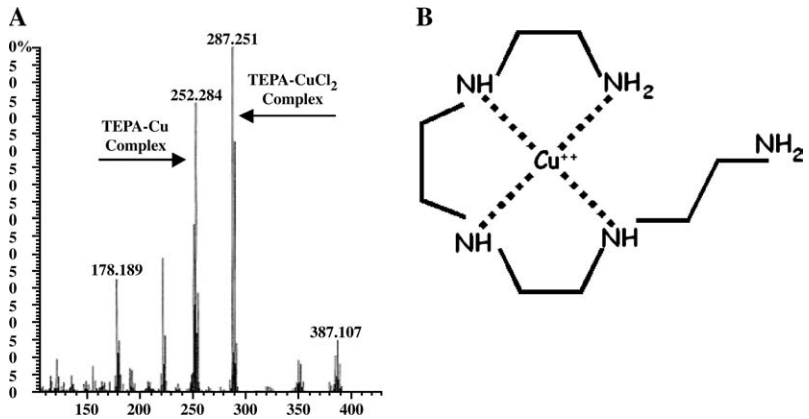

Figure 5. Mass spectrum analysis of the TEPA-Cu complex. Mass spectrum analysis of TEPA-Cu complex maintained in solution **(A)** and the two-dimensional structure of the TEPA-Cu complex **(B)** are shown.

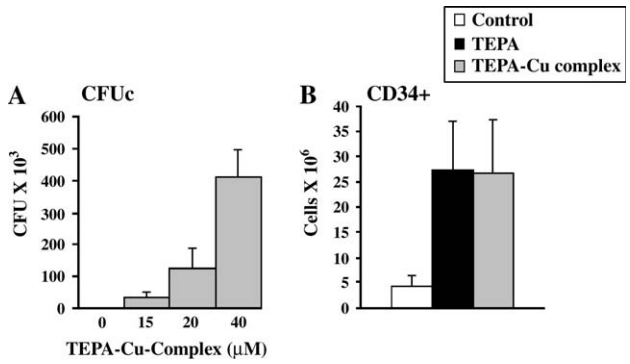

Figure 6. The biological activity of the TEPA-Cu complex. $CD34^+$ cell cultures (n = 4), grown in the presence of cytokines, were treated for 3 weeks with the indicated concentrations of the TEPA-Cu complex. From week 3 on, the cultures were grown with cytokines alone. On week 8, cultures were analyzed for their CFUc content. Cumulative numbers are shown **(A)**. $CD34^+$ cells were treated with 40 μM TEPA-Cu complex, 10 μM TEPA, or cytokines alone for 3 weeks. From week 3 on, all cultures were treated with cytokines only. At week 8, $CD34^+$ cells were repurified and enumerated (n = 4). Cumulative numbers are shown **(B)**.

atomic absorption spectrophotometry as described in Materials and methods. This technique measures total cell-associated Cu and does not discriminate between chelatable and tightly bound Cu. The results indicated that TEPA, at 5 and 10 μM, reduced overall cellular Cu by 20% and 40%, respectively, whereas the TEPA-Cu complex (25–100 μM) or Cu-chloride (5–20 μM) resulted in a dose-related increase in overall cellular Cu (Fig. 8A–C).

To determine the effect of the different culture conditions on the chelatable Cu pool, cells were loaded with CA-AM, as described in Materials and methods, followed by a 3-hour incubation with TEPA, TEPA:Cu (1:1), TEPA-Cu complex, Cu chloride, 20 μM each, or none (control). Cellular fluorescence was measured before the incubation with the above reagents and 3 hours later. The results show that the fluorescence of control cells following 3-hour incubation dropped by about 50% while the decrease in fluorescence of cells treated with TEPA, TEPA-Cu (1:1), or the TEPA-Cu complex dropped by 20% only (Fig. 9A).

Thus, the overall cellular Cu content was profoundly elevated by treatment with TEPA:Cu (1:1), TEPA-Cu complex, and Cu, while TEPA reduced it (Fig. 9B). In contrast, the chelatable Cu pool was reduced by all the TEPA reagents, while Cu chloride significantly increased it (Fig. 9A).

Discussion

In vitro expansion of HSPCs is constrained by commitment and differentiation [20]. In order to maximize the ex vivo expansion of HSPCs for research and therapeutic (transplantation) purposes attempts are constantly being made to overcome this limitation by defining epigenetic modulators that favor HSPC self-renewal with only limited differentiation [21–23]. We have previously reported data suggesting that cellular Cu content modulates self-renewal and differentiation of HSPCs [11]. Short-term (3 weeks) treatment with the Cu chelator TEPA resulted in enrichment of cord blood–derived progenitor subsets ($CD34^+CD38^-$ and $CD34^+CD38^-Lin^-$) that displayed prolonged ex vivo expansion of CFUc and $CD34^+$ cells and an enhanced capacity to repopulate NOD/SCID mice [12,13]. In contrast, 3-week treatment with Cu chloride resulted in a marked decrease in $CD34^+$ cells and the early subsets. During the treatment with Cu chloride the number of TNC and CFUc were comparable to control cultures, but the long-term potential of these cultures was impaired.

The results of these experiments suggested that changes in the cellular Cu mediated the biological effects of these

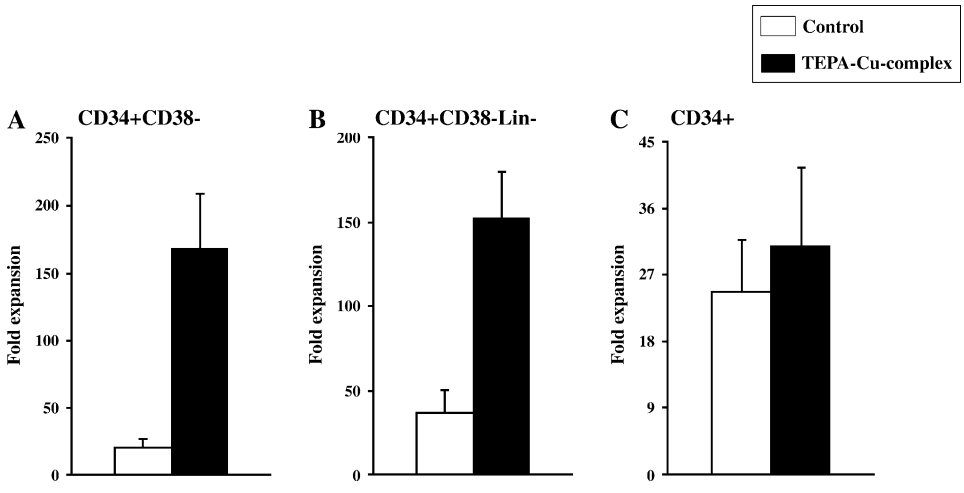

Figure 7. The effect of the TEPA-Cu complex on 3-week expansion of $CD34^+$ cell subsets. $CD34^+$ cells were treated with the TEPA-Cu complex (40 μM) or with cytokines alone. Purified $CD34^+$ cells were stained for CD34/CD38 **(A)** and CD34/Lin/CD38/L in **(B)** and analyzed by FACS. $CD34^+$ cells are shown in **C** (n = 3). Fold expansion was calculated as described in the Materials and methods.

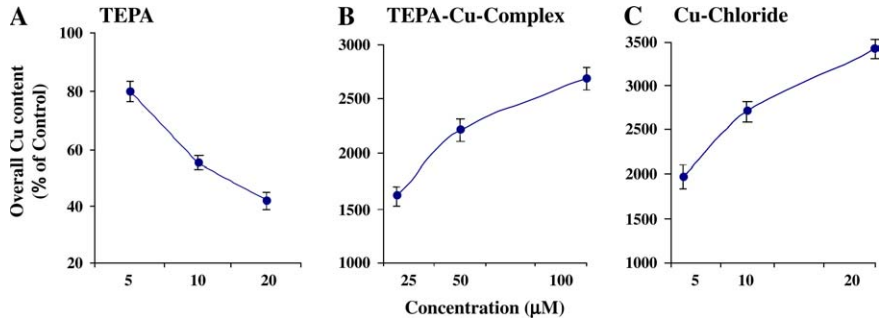

Figure 8. Measurement of overall cellular Cu content. $CD34^+$ cells were treated with the indicated concentrations of TEPA **(A)**, the TEPA-Cu complex **(B)**, Cu chloride **(C)**, or cytokines alone (control). Cellular Cu was determined by atomic absorption spectrophotometry as described in Materials and methods. Results of the Cu content are shown as percentages of control, cytokine-treated cultures.

reagents. Indeed, we demonstrated that only Cu, but not other transitional metal ions, could reverse the TEPA's effect [11]. However, this reversal was achieved only with excess of Cu. Contrary to studies in other cell systems [24,10], in our experiments, at equimolar concentration (TEPA:Cu 1:1), Cu did not quench the TEPA's effect. These surprising results prompted us to reevaluate the role of Cu in self-renewal and differentiation. For this purpose, we synthesized a stable TEPA-Cu complex at 1:1 molar ratio and evaluated its effect on CD34 cells. The results indicated that the complex had biological activity similar to the TEPA:Cu (1:1) mixture as well as TEPA.

We next determined the effect of these reagents on the overall Cu content of CD34 cells using atomic absorption spectrophotometry. The results indicated that TEPA reduced it whereas the TEPA-Cu mixture and complex or Cu chloride increased it (Figs. 8 and 9B).

Cu is present in cells in at least two various forms: one which is firmly bound to compounds such as ceruloplasmin and various enzymes such as Cu/Zn superoxide dismutase and the other a more loosely bound, labile pool, possibly involved in the synthesis of Cu proteins [25,26]. Under physiological conditions the amount of the labile pool is quite small [26], but it may have a significant biological role. To measure the cytosolic pool of labile Cu we utilized a novel flow cytometry method that makes use of a cell-permeable Cu chelator: calcein. The change in calcein fluorescence under different culture conditions measures the amount of calcein-bound Cu that in turn reflects the relative levels of cellular chelatebale Cu. We demonstrated that a 3-hour treatment with TEPA, TEPA:Cu (1:1), and TEPA-Cu complex of calcein preloaded cells reduced by 50% the decrease in fluorescence, relative to the decrease in the fluorescence of untreated cells (Fig. 9A), suggesting

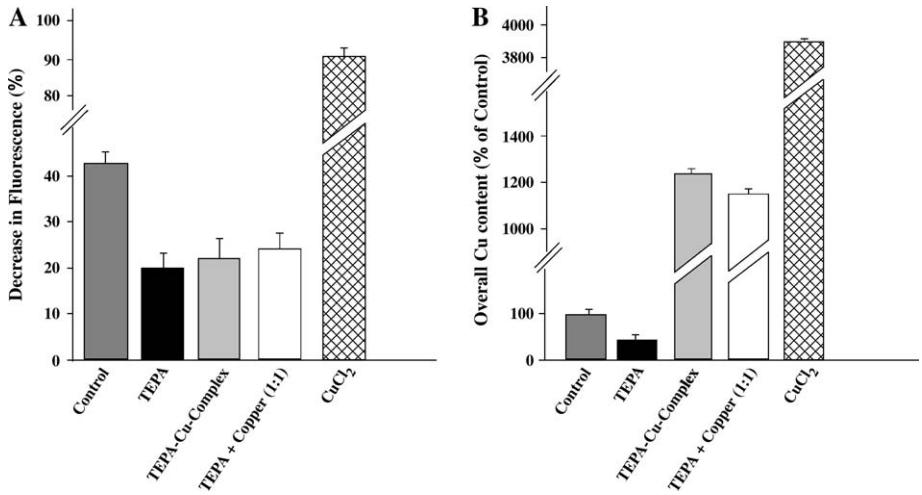

Figure 9. The effect of TEPA, TEPA:Cu (1:1), TEPA-Cu complex, and Cu chloride on chelatable **(A)** and overall **(B)** cellular Cu. Cells were loaded with calcein, then washed twice and exposed for 3 hours to either TEPA, the TEPA:Cu (1:1) mixture, the TEPA-Cu complex, and Cu chloride (20 μM each) or none (control), as indicated. Cellular fluorescence was measured after incubation with calcein (T0) and 3 hours thereafter (T3) by flow cytometry as described in Materials and methods. Percentage decrease in fluorescence during 3-hour incubation was calculated relative to the fluorescence of calcein-loaded cells (T0) as follows: fluorescence at T3 × 100/fluorescence at T0 **(A)**. The decrease in fluorescence represents, in relative terms, the available chelatable Cu pool. In parallel, overall Cu content of cells treated with 20 μM of the above-mentioned reagents was measured by atomic absorption spectrophotometry as described in Materials and methods **(B)**.

that all these reagents reduce the availability of chelatable Cu. Thus, although the overall cellular Cu content was profoundly elevated by treatment with TEPA:Cu (1:1) mixture and complex, as well as Cu chloride, and TEPA reduced it, the chelatable Cu was reduced by all the TEPA reagents, while Cu chloride significantly increased it.

At least two mechanisms may account for the biological effects of TEPA; the first suggests that a TEPA-Cu chelate is the specific active intermediate responsible for TEPA's biological activity. Since TEPA has the strongest binding affinity for Cu (its stability constants for Cu, Zn, Co, Fe, Mn are 23, 15, 13, 10, 7, respectively) [27], it is expected to form a chelate, similar to the synthesized TEPA-Cu complex [25]. The chelate, as well as the complex, acts directly to affect pathways involved in self-renewal and differentiation of CD34 cells. This mechanism does not require reduction of cellular Cu since the TEPA:Cu (1:1) mixture and the TEPA-Cu complex, which display biological activity similar to TEPA, have opposite effect on the overall Cu content. This mechanism also fails to explain the effect of Cu chloride on self-renewal and CD34 cell differentiation. The second mechanism involves changes in the levels of the chelateble Cu pool. All the TEPA reagents were found to reduce this pool while Cu chloride was found to increase it. This correlates with their effect on self-renewal and differentiation.

Intracellular Cu was reported to regulate gene expression [28–31] and cell differentiation [32,33] by a variety of pathways. It modifies the function of transcription factors and the activities of Cu enzymes such as S-adenosylhomocysteine hydrolase and protein arginine methyltransferase 1, which are involved in protein methylation. Cu chelation by TEPA deactivates these enzymes, resulting in protein hypomethylation and inhibition of neurite differentiation [24]. Cu has also been shown to suppress the enzyme histone acetyl transferase, resulting in a decrease in overall and specific histone acetylation; Cu chelators, in contrast, had an opposite effect [34]. Reversible histone acetylation/deacetylation plays a pivotal role in transcriptional modulation of cell fate [35,36]. Histone deacetylase inhibitors were reported to increase the self-renewal of hematopoietic $CD34^+$ cells in vitro and their engraftability in vivo [37]. Cu may be involved in hematopoietic cell regulation by modulating cellular post-translational modification activities.

The results of the present study support the notion that reduction of the chelatable Cu pool rather than a specific TEPA-Cu chelate mediates the mechanism of TEPA's activity on $CD34^+$ cells. Further studies are in progress to clarify this mechanism.

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