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The Effect of Tetraethylenepentamine, a Synthetic Copper Chelating Polyamine, on Expression of CD34 and CD38 Antigens on Normal and Leukemic Hematopoietic Cells

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We have previously found that the synthetic polyamine tetraethylenepentamine (TEPA) significantly delayed differentiation and prolonged expansion of cord-blood derived HPC in cytokine-supplemented cultures. Most HPC have the CD34+CD38+ phenotype, but the minority CD34+38− cells are primitive subset of HPC that have the potential for long-term repopulation *in vivo*. We investigated the effect of TEPA on the CD34/CD38 surface antigen expression of human myeloid leukemia cell lines as well as normal cord blood derived hematopoietic cells. Confirming previous results, our data showed that both the leukemic and normal cells increased their CD38 expression when grown in serum-containing medium or when treated with retinoic acid. In the present study, we found that TEPA inhibited CD38 under these conditions in both normal and leukemic cells. As for CD34, TEPA increased the proportion of CD34 cells in short- and long-term normal cultures but not in the leukemic cell lines. These results suggest that *ex vivo* expansion of HPC depends on the presence of CD34+CD38− cells and that TEPA prolongs HPC expansion by inhibiting the CD38− to CD38+ transition.

Keywords: Hematopoietic stem cells; Retinoic Acid; Proliferation; Differentiation; Leukaemia

INTRODUCTION

Self-renewal of hematopoietic stem and progenitor cells (HPC) both *in vivo* and *in vitro* is limited by cell differentiation. We have previously found that the synthetic polyamine tetraethylenepentamine (TEPA) significantly prolonged expansion of HPC in cytokine-supplemented, stroma-free liquid cultures initiated with cord-blood derived HPC [1]. For this long-term effect, TEPA had to be present at the early stages (3 weeks) of the cultures, after which its presence could be discontinued without affecting its ability to prolong the active proliferation period of the cultures. We have also shown that this effect on cell proliferation was associated with inhibition of cell differentiation as measured by morphology, cloning potential, surface antigens and differentiated cell functional activities [1].

Differentiation in the hematopoietic system involves, among other changes, altered expression of surface antigens [2]. Thus, changes in the CD34 and CD38 antigens underlie the process of differentiation of stem cells [3]. Most of the pluripotent hematopoietic stem cells and lineage committed progenitor cells carry the CD34 antigen that is gradually lost upon differentiation. The CD38 antigen is present on most, but a few, stem cells [3]. Experiments in SCID mice suggested that cells with the CD34+CD38− phenotype are a primitive subset of the stem cell pool that has the potential for long-term repopulation [4].

Several agents, such as interleukin-4, interferon-gamma [5] and especially retinoids [6–8] have been reported to increase CD38 expression, but so far no agent has been reported to decrease the antigen expression or to maintain it in hematopoietic differentiating cells.

We now report on the effect of TEPA on CD34 and CD38 surface antigen expression. We used human myeloid leukemia *in vitro* established cell lines (two early myeloblastic CD34+CD38+ cell lines, ML-1 [9] and KG-1a [10], two CD34−CD38+ myeloblastic/promyelocytic cell lines, HL-60 [11] and CB-1, as well as normal hematopoietic cells derived from neonatal cord blood (CB). Cells were cultured in serum-free or serum-containing media either with or without all-trans retinoic acid (ATRA) and the antigens were measured

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by flow cytometry during the first 2 weeks of the cultures.

Confirming previous results [12], our present data showed that both leukemic or normal hematopoietic cells increased their CD38 expression when grown in serum-containing medium or treated with ATRA. In the present study we showed that TEPA inhibited CD38 expression; this effect was observed both in serum-containing or serum-free cultures, either in the presence or absence of ATRA. No effect of TEPA was noted on CD34 expression in either the CD34+ or CD34− leukemic cell lines tested. As for normal cell cultures, the inhibitory effect of TEPA on CD38 expression was accompanied by maintenance of the CD34 phenotype, thus, generating a higher proportion of total CD34 population and especially increased CD34+CD38− population following treatment with TEPA. These results suggest that *ex vivo* expansion of HPC is dependent on the presence of a sub-set of cells with CD34+CD38− cells in the population and that the effect of TEPA on long-term self-renewal in CD34+ cultures involves inhibition of the CD38− to CD38+ transition.

MATERIALS AND METHODS

Cells and Culture Conditions

The ML-1 cell line (purchased from American Type Cell Collection (ATCC), CRL-11451) was established by growing CD34+ cells, immuno-selected from bone marrow from human cadaveric vertebral bodies [9]. The KG-1a cell line (ATCC, CCL 246.1) was established from the bone marrow of a patient with acute myelogenous leukemia. Cells of the original line, termed KG-1, have the morphology of myeloblasts and they spontaneously differentiate to granulocyte- and macrophage-like cells and show a good response to colony stimulating factor [13]. The variant KG-1a cells are morphologically, cytochemically and functionally less mature than the parental KG-1. They have the morphology of undifferentiated promyeloblasts, do not spontaneously differentiate to granulocyte- and macrophage-like cells, do not express the DR antigen and do not respond to colony stimulating factors or phorbol esters [10]. The HL-60 cell line was originally established from a patient with acute promyelocytic leukemia [11].

The CB-1 cell line was established in our laboratory by growing CD34+ cells immuno-selected from human neonatal CB. Upon injection of these cells into SCID mice leukemia was developed within 1 month. Cell lines were maintained by sub-culturing twice weekly at about 1×10^5 cells/ml in alpha-minimal essential medium supplemented with 10% fetal bovine serum (both from Biological Industries, Beit-Hamek, Israel) and incubating at 37°C in a humidified atmosphere of 5% CO_2 in air. Some experiments were performed in serum-free medium—alpha medium supplemented with 20 μl/ml of Biogro 2 (Biological Industries) and 1% bovine serum albumin.

Normal cultures were established by cells obtained from fresh neonatal umbilical CB. In both cases, CD34 cells were purified (> 95%) by immuno-magnetic beads (Miltenyi Biotec, Auburn, CA) according to the manufacturer's instructions. The cells were washed and cultured at 10^4 cells/ml in alpha medium supplemented with 10% fetal bovine serum and the following cytokines: Stem cell factor, thrombopoietin, interleukin-6 and FLt-3 ligand (all from Pepro Tech, Inc. Rocky Hill, NJ, at 50 ng/ml each). At weekly intervals, cultures were semi-depopulated and supplemented with fresh medium, serum and cytokines. Some experiments were performed with CB-derived cells negatively selected against 12 lineage specific cell surface antigens including CD38 (StemCell Technologies, Vancouver, Canada). This procedure yields a highly purified population of CD34+CD38− cells [14].

In some experiments cells were exposed to *all-trans* retinoic acid (ATRA) (Sigma) dissolved at 10^{-3} M in ethanol, tetraethylenepentamine (TEPA) (Aldrich Chemical Co., WI) dissolved at 10 mM in water and to zinc sulfate (#Z-0251, Sigma), ferric ammonium citrate or cupric sulfate (#C-6283, Sigma), 10 μM each.

Immunophenotyping

For immuno-phenotyping, cells were harvested, washed with phosphate buffered saline, containing 1% bovine serum albumin and 0.1% sodium azide, and stained by incubation (at 4°C for 60 min) with phycoerythrin (PE)-conjugated anti CD38 antibodies (Immunoquality Products, The Netherlands) and fluorescein isothiocyanate (FITC)-conjugated anti CD34 antibodies (Miltenyi Biotec) both at 5 μl per 50 μl cell suspension. The cells were then washed with the same buffer and analyzed, using FACS-calibur flow cytometer (Becton-Dickinson, Immunofluorometry systems, Mountain View, CA). Cells were passed at a rate of about 1,000 cells/second, using saline as the sheath fluid. A 488 nm argon laser beam served as the light source for excitation. Emission of 10,000 cells was measured using logarithmic amplification and calculated using the CellQuest software. Background noise was determined using isotype control stained cells. Arithmetic Mean Fluorescence Channels (MFC) of the negative and positive populations and the stained cells to noise (S/N) ratio were calculated.

The results are presented as the mean value ± SD of 4 experiments.

RESULTS

Effect of TEPA on Human Myeloid Cell Lines

In KG-1a and ML-1 cell cultures, more than 95% of the cells were positive for CD34; 66.7 and 28.5% of the cells were positive for CD38 (with a MFC of 138.8 and 62.1), respectively. HL-60 and CB-1 cells were negative for CD34; 70 and 90% of the cells were positive for CD38

(with MFC of 43 and 150), respectively. When these cell lines were cultured for 3 days in the presence of TEPA (0.1 – 1 mM) no effect was observed on CD34 expression (data not shown), however, as depicted in Fig. 1, CD38 expression was inhibited. Longer incubation with TEPA did not further change the effect (data not shown).

We have previously shown that retinoic acid (ATRA) significantly increased the expression of CD38 in these cell lines [15]. We, therefore, analyzed the effect of TEPA on CD38 in leukemic cells treated with different concentrations of ATRA. The results show a dose-dependent increase of CD38 by ATRA and inhibition of this increase by TEPA (Fig. 2).

The above described experiments were carried out in serum-supplemented medium. Since serum has been reported to increase CD38 expression [12], we studied the effect of TEPA on cells grown either with or without serum. The results showed a significant decrease of CD38 expression in all these cell lines when grown in serum-free medium as compared to serum-containing medium. TEPA further reduced CD38 expression of all the cell lines in serum-containing medium and of ML-1, HL-60 and CB-1 cells, but not of KG-1 cells, in serum-free medium (Fig. 3).

Effect of TEPA in Cultures Initiated with Cord Blood-Derived Purified CD34+ Cells

We further determined the effect of TEPA in cultures initiated with CB-derived CD34 cells. Fig. 4 depicts flow cytometric dot-plots with respect to CD34 and CD38 expression of cultures maintained for 3 days in the presence of early acting cytokines with or without TEPA in either serum-free or serum-containing medium. In serum-containing, TEPA-free, cultures 44.7% (CD38 – MFC – 180) of the cells were CD34 – CD38 +, 50.5% (CD38 – MFC – 92.3) were CD34 + CD38 + and 3.2% were CD34 + CD38- (Fig. 4A). In serum-containing medium, TEPA (20 μM)-containing, cultures 19.0% (CD38 – MFC – 154.1) of the cells were CD34 – CD38 +, 65.0% (CD38 – MFC – 66.4) were CD34 + CD38 + and 13.2% were CD34 + CD38 – (Fig. 4B). Maximal effect was observed with 50 μM TEPA where the percentage of CD34 + CD38- cells increased from 3.2% in TEPA-free to 17.6% in TEPA-containing cultures; this was accompanied by a reciprocal decrease in the percentage of CD34 + CD38 + cells.

In serum-free, TEPA-free, cultures 22.4% (CD38 – MFC – 184) of the cells were CD34 – CD38 +, 45.3% (CD38 – MFC – 87.2) were CD34 + CD38 + and 27.9% (CD38 – MFC – 14.8) were CD34 + CD38 – (Fig. 4C). In serum-free, TEPA-containing, cultures 21.1% (CD38 – MFC – 258.3) of the cells were CD34 – CD38 +, 38.9% (CD38 – MFC – 78.6) were CD34 + CD38 +, and 33.9% (CD38 – MFC – 13.4) were CD34 + CD38- (Fig. 4D). Thus, the CD34 + CD38 – population increased from 3.2% in serum-containing, TEPA-free medium to 27.9% in serum-free, TEPA-free, medium up to 33.9% in serum-free, TEPA-containing, medium.

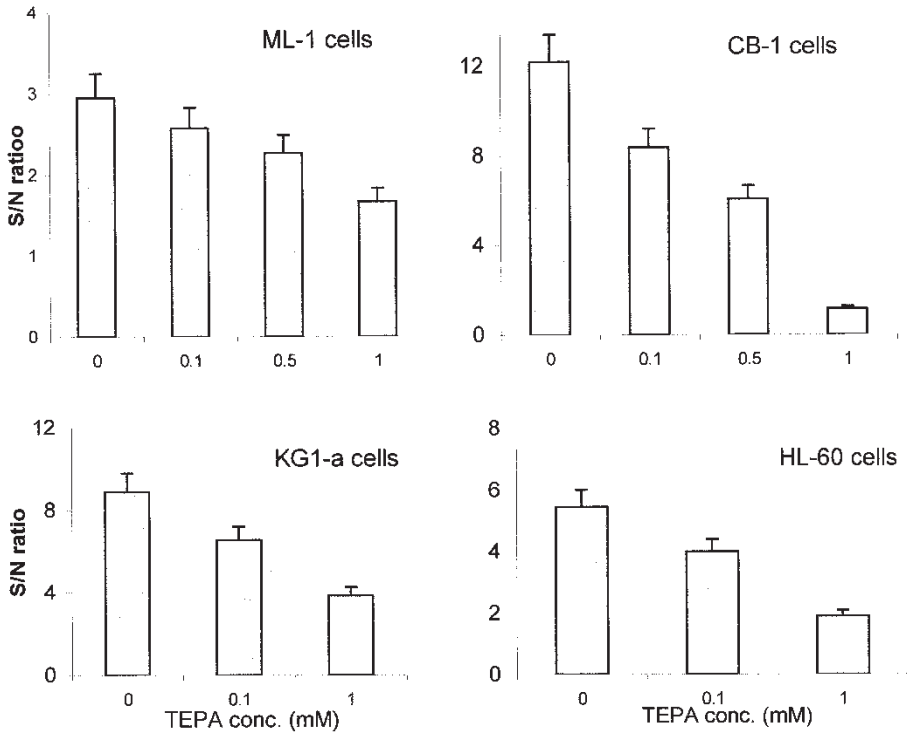

FIGURE 1 The effect of TEPA on CD_{38} expression by human myeloid leukemic cell lines. Cells were cultured with the indicated concentrations of TEPA and stained, after 3 days, with anti-CD38 antibodies as described in Materials and Methods. The results are expressed as the ratio (S/N) of the arithmetic Mean Fluorescence Channels of the stained cells to noise.

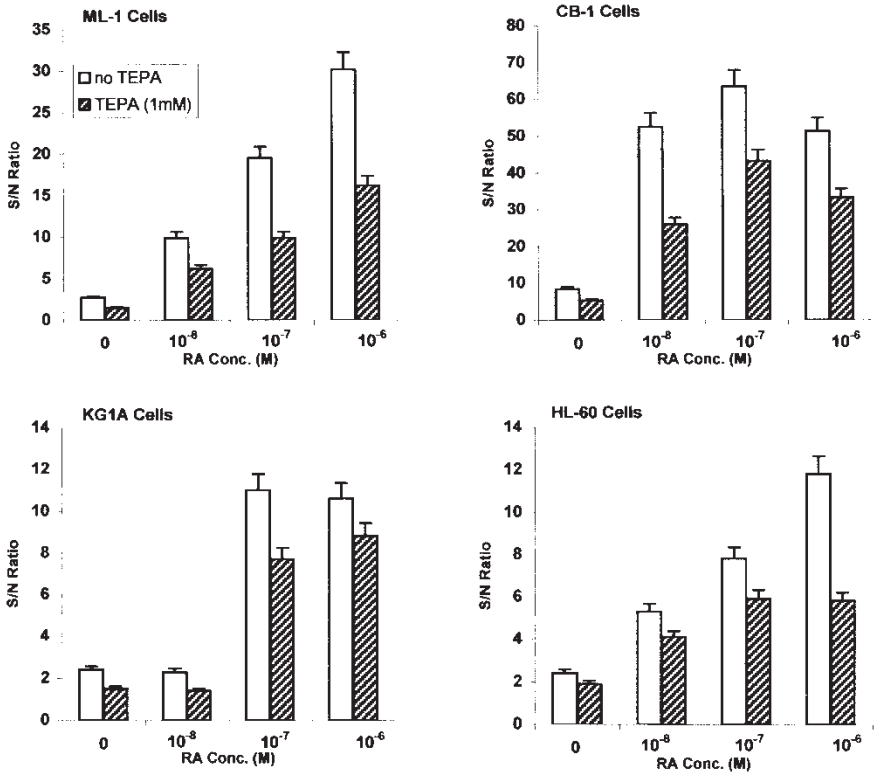

FIGURE 2 The effect of TEPA on stimulation by retinoic acid of CD38 expression by human myeloid leukemic cell lines. Cells were cultured with 1 mM TEPA and the indicated concentrations of ATRA (10^{-8} to 10^{-6} M) and stained, after 3 days, for CD38. The results are expressed as the S/N ratio.

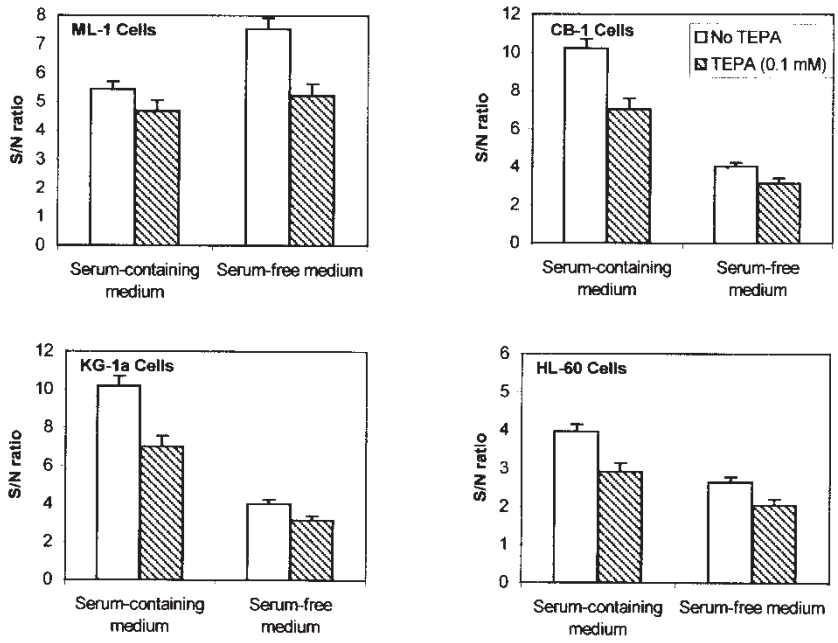

FIGURE 3 The effect of TEPA and serum on CD38 expression by human myeloid leukemic cell lines. The indicated cell lines were cultured with or without TEPA (0.1 mM) for 3 days either in serum-containing or serum-free medium. The results are expressed as the S/N ratio.

Fig. 5 shows the effect of TEPA, at 20 and 50 μM, on the expression of CD34 and CD38 in day 3 and in day 6 cultures. The percentages of (%) CD34+ cells and the %CD34+CD38− cells in the total cell population, and %CD38− cells in the CD34+ cell population were calculated. The results showed that all these parameters decreased with time. In TEPA-treated cultures, these percentages were maintained at higher levels compared to control cultures, while the CD34 expression of cells (the MFC of CD34 expression) decreased. Although the

proportions of CD34+ cells and the %CD34+CD38− cells in the total cell population decreased, their absolute number increased, especially in the presence of TEPA [1] (not shown).

Fig. 6 summarizes the effect of TEPA on CD38− cells in 3 day and in 6 day serum-containing or serum-free cultures. The results show that three parameters affect the %CD38− cells: Time in culture, the presence of serum and the presence and concentration of TEPA. Similar results were obtained in cultures initiated with highly

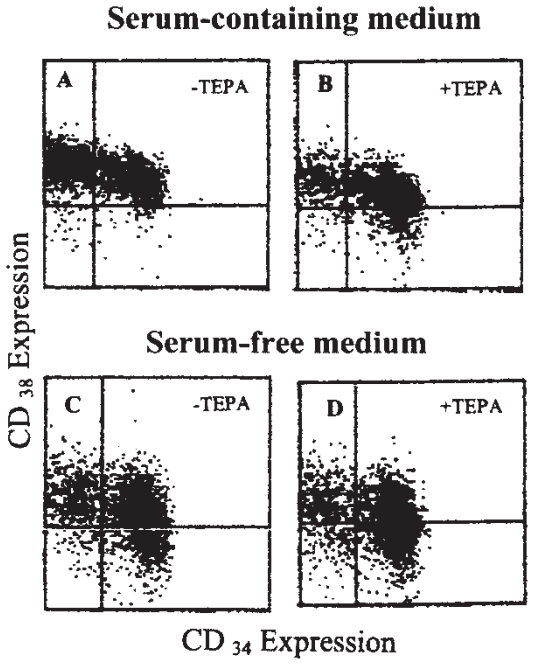

FIGURE 4 Flow cytometric analysis of the effect of TEPA on CD34 and CD38 expression of cord blood derived CD34+ cells cultured with or without serum. Cells were cultured for 3 days in serum-containing (A, B) or serum-free (C, D) medium without (A, C) or with (B, D) 20 μM TEPA.

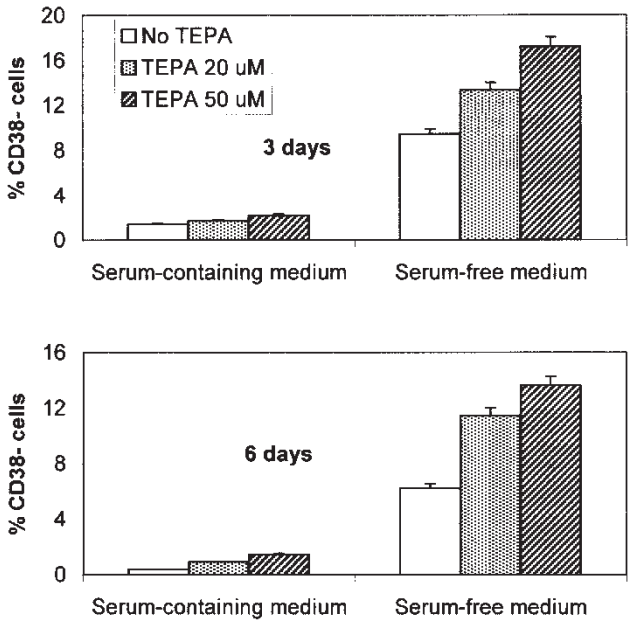

FIGURE 6 The effect of TEPA on CD38 expression on cord blood derived CD34+ cells cultured in serum-containing medium and in serum-free medium. The percentages of cells with the CD34+CD38− phenotype after 3 or 6 days of culture are shown.

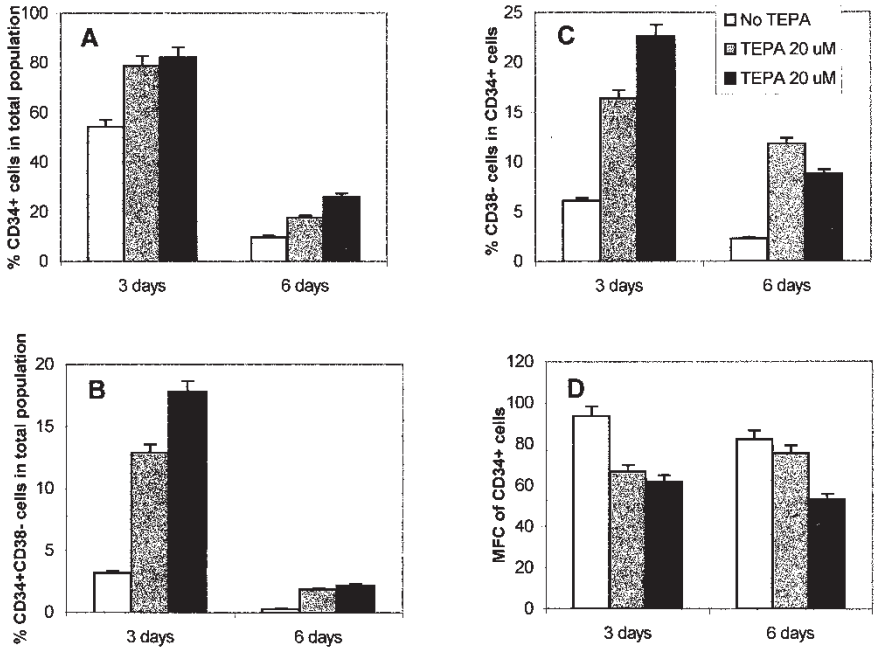

FIGURE 5 The effect of TEPA on CD34 and CD38 expression in cord blood-derived CD34+ cell cultures. Cells were grown for 3 or 6 days with the indicated concentrations of TEPA. The results are expressed as the percentage of CD34+ cells (both CD38+ and CD38−) in the total population (A), the percentage of CD34+CD38− cells in the total population (B), the percentage of CD38− cells in the CD34+ population (C) and the MFC of the CD34+ cell population (D).

purified population of CB-derived CD34+CD38− cells by negative selection (data not shown).

To determine whether the TEPA effect is mediated through its Cu-binding capacity, CB-derived CD34+ cell cultures were grown in the presence of various transition metal ions and TEPA. Fig. 7 shows that only Cu was able to override the TEPA effect on CD34 expression.

DISCUSSION

Most of the normal human hematopoietic pluripotent stem cells and the lineage committed progenitor cells (HPC) are CD34+. The majority is CD34+CD38+, with a minority ($< 10\%$) being CD34+CD38− [3]. The CD34+CD38− phenotype appears to identify the most immature hematopoietic 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 [16] and exhibits longer maintenance of their phenotype [17] and delayed proliferative response to cytokines as compared to CD34+CD38+ cells [18]. CD34+CD38− cells can give rise to lymphoid and myeloid progeny *in vitro* [19] and have an enhanced capacity to repopulate immunodeficient (SCID) mice [4]. Moreover, in patients who received autologous stem cell transplantation, the number of CD34+CD38− cells infused correlated positively with the speed of hematopoietic recovery [20]. In line with these functional features, CD34+CD38− cells have been shown to have detectable levels of telomerase [21].

We have previously found that depletion of copper by polyamine chelators, such as TEPA, transiently blocked differentiation, thus, allowing increased and prolonged cytokine-supported expansion of HPC with minimal cell differentiation [1]. In the present study we investigated the effect of TEPA on CD34 and CD38 surface antigen expression. For this purpose, we used human myeloid leukemia *in vitro* established cell lines as well as normal hematopoietic cells derived from neonatal CB. Cells were cultured in serum-free or serum-containing medium either with or without ATRA, a well-known inducer of CD38 expression [6–8], and the antigens were measured by flow cytometry during the first 2 weeks of the cultures.

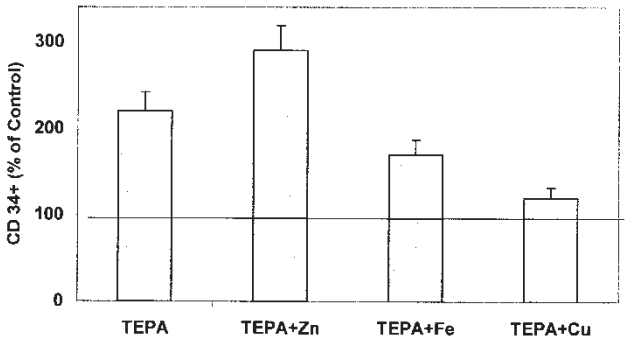

FIGURE 7 The effect of TEPA and various metal ions on CD34 expression on cord blood derived CD34+ cells. Cells were cultured for 3 days with 20 μM TEPA alone or in addition to zinc sulfate, ferric ammonium citrate or cupric sulfate, 10 μM each. CD34+ cells, as % of control, are shown.

In either the CD34+ or CD34− leukemic cell lines, we did not find any effect of TEPA on CD34 expression. In contrast, TEPA inhibited CD38 expression in cultures grown in the presence or absence of ATRA in either serum-containing or serum-free medium. As for normal cultures initiated with CB-derived CD34 purified cells, TEPA increased CD34 and decreased CD38 expression, thus, generating a higher number CD34 cells and increasing the proportion of CD34+CD38− cells compared to untreated cultures.

This effect of TEPA can most probably be attributed to its copper binding capacity, as we have previously shown that it decreased cellular copper content in CD34 cultures [1]. Indeed, addition of excess copper, but not other metal ions overrides the TEPA effect. Furthermore, ceruloplasmin, the main copper-binding protein in the serum, had an opposite effect to TEPA; it caused a decrease in the percentage of CD34 and an increase in CD38 cells (data not shown).

Ex-vivo expansion of HPC may have important clinical applications in cell therapy. We have previously shown that cultures of CB-derived purified CD34 cells grown in serum and cytokine-supplemented liquid medium continued to expand for 8 weeks, but eventually ended upon differentiation of the cells into non-dividing mature cells, mostly macrophages and granulocytes, suggesting that cell proliferation in this system was limited by terminal cell differentiation. TEPA significantly increased the length and extent of expansion. The present results suggest that *ex vivo* expansion of HPC is dependent on the presence of a sub-set of cells with CD34+CD38− phenotype and that the effect of TEPA on long-term self-renewal in CD34 cultures involves inhibition of the CD38− to CD38+ transition.

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