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Cellular copper content modulates differentiation and self-renewal in cultures of cord blood-derived $CD34^+$ cells

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Summary. Several clinical observations have suggested that copper (Cu) plays a role in regulating haematopoietic progenitor cell (HPC) development. To further study this role we used an *ex vivo* system. Cord blood-derived $CD34^+$ cells were cultured in liquid medium supplemented with Kit-ligand, FLt3, interleukin 6 (IL-6), thrombopoietin and IL-3. Under these conditions, Cu content, measured by atomic absorption, was 7 ng/10^7 cells. Modulation of intracellular Cu was achieved by supplementing the cultures with the Cu chelator tetraethylenepentamine, which reduced cellular Cu (4 ng/10^7 cells), or ceruloplasmin or Cu sulphate that elevated cellular Cu (18 and 14 ng/10^7 cells respectively). The results indicated that low Cu content delayed differentiation, as measured by the surface antigens CD34, CD14 and CD15, colony-forming unit (CFU) frequency and cell morphology, while high Cu accelerated differentiation compared with Cu unmanipulated cultures. As a result, expansion of total cells, CFU and $CD34^+$ cells in low Cu was extended (12–16 weeks), and in high Cu was shortened (2–4 weeks), compared with control cultures (6–8 weeks). These effects required modulation of intracellular Cu only during the first 1–3 weeks of the culture; the long-term effects persisted thereafter, suggesting that the decision process for either self-renewal or differentiation is taken early during the culture. This novel method of controlling cell proliferation and differentiation by copper and copper chelators might be utilized for *ex vivo* manipulation of HPC for various clinical applications.

Keywords: stem cells, proliferation, differentiation, copper, tetraethylenepentamide.

The possible involvement of copper (Cu) in haematopoietic stem and progenitor cell (HPC) development can be inferred from Cu-deficiency situations as a result of either hereditary defects, e.g. Menkes syndrome or Celiac disease, or acquired conditions (Cordano *et al*, 1966). The latter is typically associated with malnourishment such as total parenteral nutrition without Cu supplementation, consumption of high levels of zinc (Botash *et al*, 1992), which interferes with Cu utilization, and in underweight and/or cow milk-fed (poor source of Cu) newborns, which may result in severe cases in Shwanchman syndrome. Unbalanced treatment with Cu chelators in Cu-overload cases such as occur in Wilson's disease may also lead to Cu deficiency (Dubois *et al*, 1990).

The clinical symptoms of Cu deficiency may include various systems. Of particular relevance to this study is the fact that Cu deficiency is often associated with haematopoietic cell differentiation arrest, which results in anaemia, neutropenia and thrombocytopenia. All these pathological manifestations are unresponsive to iron therapy, but are rapidly reversed following Cu supplementation (Zidar *et al*, 1977; Hirase *et al*, 1992; Banno *et al*, 1994; Wasa *et al*, 1994).

Morphological examination of the bone marrow (BM) of neutropenic Cu-deficient patients demonstrated the absence of mature cells ('maturation arrest'). The presence of immature myeloid progenitors was confirmed by cloning cells from Cu-deficient patients in semisolid medium containing normal serum. No colonies developed in Cu-deficient serum. These results indicated the presence of intact progenitors in the patient's BM, and suggested that the block in development occurs distal to the progenitor stage (Zidar *et al*, 1977).

The objective of the present study was to investigate the involvement of cellular Cu in the regulation of HPC proliferation and differentiation. For this purpose we used cultures of cord blood (CB)-derived purified CD34 cells grown in cytokine-supplemented liquid medium. Cellular Cu was modulated, either increased or decreased, by adding Cu or Cu cheletors, tetra-ethylenepentamine (TEPA) and ceruloplasmin (Cp), to the culture medium. Our findings indicated that, under reduced Cu conditions (TEPA-supplemented cultures), HPC

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differentiation was delayed resulting in extended active cell proliferation, whereas under elevated Cu conditions (Cu sulphate- or Cp-supplemented cultures), differentiation was accelerated resulting in a shorter phase of cell proliferation.

MATERIALS AND METHODS

Cell collection and purification. Cells were obtained from umbilical CB after a normal full-term delivery (informed consent was given). Samples were collected and processed within 12 h post partum. Blood was mixed with 3% Gelatin (Sigma, St. Louis, MO, USA) and sedimented for 30 min to remove most of the red blood cells (RBC). The leucocyte-rich fraction was harvested and layered on Ficoll-Hypaque gradient (1·077 g/ml; Sigma), and centrifuged at 400 **g** for 30 min. The mononuclear cells in the interface layer were then collected, washed three times and resuspended in phosphate-buffered saline (PBS) (Biological Industries Beit-Hamek, Israel) containing 0·5% bovine serum albumin (BSA) (Fraction V; Sigma). To purify the $CD34^+$ cells, the mononuclear cell fraction was subjected to two cycles of immuno-magnetic separation using the 'MiniMACS CD34 progenitor cell isolation kit' (Miltenyi Biotec, Aubun, CA, USA) following the manufacturer's recommendations. The purity of the $CD34^+$ population obtained ranged from 95% to 98% as evaluated by flow cytometry (see below).

Ex vivo expansion of $CD34^+$ cells. Purified $CD34^+$ cells were cultured in 24-well Costar Cell Culture Clusters (Corning Inc. Corning, NY, USA) at 10^4 cells/ml in alpha medium supplemented with 10% fetal bovine serum (FBS) (Biological Industries) and the following human recombinant cytokines: thrombopoietin (TPO), interleukin 6 (IL-6) and FLT-3 ligand, at final concentration of 50 ng/ml each, and IL-3 at 20 ng/ml (all from Perpo Tech, Rocky Hill, NJ, USA). Cellular Cu content was modulated by supplementing the culture with either tetra-ethylenepentamine (TEPA) (Aldrich Chemical Co., WI, USA), ceruloplasmin or Cu sulphate (both from Sigma) as indicated. At weekly intervals, cell cultures were demi-populated and supplemented with fresh medium, serum, cytokines and other components as indicated. The cultures were incubated at 37°C in a humidified atmosphere of 5% CO_2 in air.

At various time points, cells were harvested, counted following staining with trypan blue, and cell morphology was determined on cytospin (Shandon, UK)-prepared smears stained with May–Grunwald/Giemsa solutions.

Assay for colony forming units (CFU). Cells were cloned in semisolid, methylcellulose-containing medium supplemented with 2 IU/ml erythropoietin (Eprex, Cilag AG Int., Switzerland), stem cell factor and IL-3, both at 20 ng/ml, granulocyte colony-stimulating factor (G-CSF) and granulocyte–macrophage CSF (GM-CSF), both at 10 ng/ml (all from Perpo Tech). Cultures were incubated for 14 d at 37°C in a humidified atmosphere of 5% CO_2 in air.

Surface antigen analysis. Cells were harvested, washed with PBS containing 1% BSA and 0·1% Na azide (Sigma) and stained (at 4°C for 60 min) with fluorescein isothiocyanate or phycoerythrin-conjugated antibodies (all from Immunoquality Products, the Netherlands). The cells were then washed with the same buffer and analysed using FACSCALIBUR or FACSTAR^{PLUS} flow cytometers. Cells were passed at a rate of 1000 cells/s, using saline as the sheath fluid. A 488-nm argon laser beam served as the light source for excitation. Emission of 10^4 cells was measured using logarithmic amplification and analysed using CELLQUEST software.

Copper determination. Cells were harvested and washed three times using PBS. Aliquots, containing 2×10^6 cells, were then transferred to metal-free Eppendorf tubes and pelleted. The pellets were brought to a concentration of 1×10^{10}/l cells with 0·03 mol/l ultrapure nitric acid (Mallinckrodt Baker B.V, Deventer, the Netherlands). Samples were sonicated and then analysed using a graphite furnace atomic absorption spectrophotometry model 460 with HGA 2200 controller (Perkin Elmer, Norwalk, CT, USA) at a wavelength of 324·7 nm and 0·7 nm slit width). The following times and temperatures were used: drying at 95°C for 45 s with 15-s ramp; charring at 900°C for 30 s with 10 s ramp and atomization at 900°C for 10 s. Peak area was integrated for 10 s.

Total cell counts, number of $CD34^+$ cells and CFU numbers are presented as accumulative numbers per ml culture, assuming that the cultures had not been passed; i.e. the numbers per ml were multiplied by the dilution factor. The results are presented as the mean ± SD of four determinations of a representative experiment out of three experiments performed.

RESULTS

Modulation of cellular Cu by TEPA, Cp and Cu sulphate

We first determined the effects of TEPA, Cp and Cu sulphate on cellular Cu content in $CD34^+$ cultures (Fig 1). Cord blood-derived, purified $CD34^+$ cells were grown in serum- and cytokine-supplemented medium as described in *Materials and methods*. Atomic absorption measurements indicated that this medium contained 17 ng/ml Cu. After

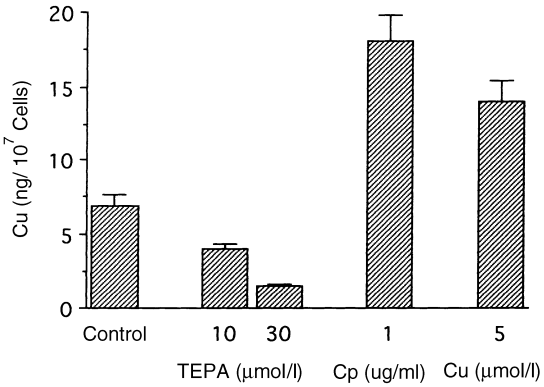

Fig 1. Effect of tetra-ethylenepentamine (TEPA), ceruloplasmin or Cu sulphate on cellular Cu content. $CD34^+$ cell cultures were supplemented for 2 d with or without the indicated concentrations of TEPA, Cu sulphate or Cp. Cellular Cu was determined by atomic absorption. The results show the mean ± SD of four determinations.

2 d in this medium, cells contained 7 ng of Cu per 10^7 cells. When the Cu chelator, TEPA, at 10 and 30 μmol/l, was included in the culture medium, intracellular Cu content dropped to 4 and 1·5 ng/10^7 cells respectively. Addition of Cp (1 μg/ml) or Cu sulphate (5 μmol/l) to the culture medium increased intracellular Cu to 18 and 14 ng/10^7 cells respectively. These results indicate that intracellular Cu content can be modulated by the addition of TEPA, Cp or Cu sulphate to the culture medium.

Modulation of cellular Cu: effect on cell proliferation

In order to test the effect of Cu modulation on long-term cell proliferation, $CD34^+$ cultures were maintained continuously in the presence of TEPA, Cp or Cu sulphate; at weekly intervals, the cultures were demi-depopulated and supplemented with half the volume of fresh medium, serum, cytokines and TEPA, Cp or Cu sulphate. Figure 2 depicts the kinetics of cell accumulation in such cultures. Determination of cell number and CFU content at week 7 (Fig 3) revealed that cultures treated continuously with TEPA (at 5–15 μmol/l) (Fig 3A) contained higher numbers of total cells and CFU, and cultures treated continuously with Cu sulphate (1–10 μmol/l) or Cp (0·1–5 μg/ml) (Fig 3B) contained lower numbers of such cells compared with control cultures. At high concentrations (> 15 μmol/l), TEPA reduced cell and CFU numbers (Fig 3A). No difference among the cultures was noted with respect to number of total cells and number of CFU during the first 3 weeks (data not shown).

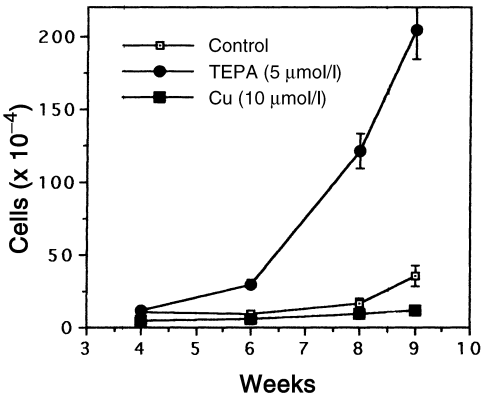

Fig 2. The effect of tetra-ethylenepentamine (TEPA) and Cu sulphate on the kinetics of cell accumulation in $CD34^+$ cell cultures. $CD34^+$ cell were grown continuously in the presence of TEPA (5 mmol/l), Cu sulphate (10 mmol/l) or none. The cultures were split weekly using a 1:2 dilution of fresh medium, serum, cytokines and TEPA or Cu sulphate. Viable cells were determined following staining with trypan blue. Results are presented as accumulative numbers; i.e. the numbers of cells present per ml culture multiplied by the dilution factor.

Fine-tuning of cellular Cu could be achieved by adding both TEPA and Cu, at various concentrations. Figure 4 shows the accumulative number of total cells after 9 weeks in culture. The results show that 5 μmol/l TEPA (TEPA 5, Cu 0) increased cell number by sixfold over controls (TEPA 0, Cu 0), while 10 μmol/l Cu sulphate (TEPA 0, Cu 10) decreased it by 30%. Simultaneous addition of both reagents (TEPA 5, Cu 10) reduced the TEPA effect to only 9% of that of TEPA alone. Thus, Cu sulphate, at 10 μmol/l, abolished the stimulating activity of TEPA at its' optimal concentration (5 μmol/l). This reversal of the stimulating activity of TEPA was restricted to Cu; other metal ions such as zinc, selenium and iron (Fig 5) or calcium and magnesium (data not shown) failed to reverse the effect. In contrast to the inhibitory effect of Cu on optimal concentrations of TEPA, Cu restored the stimulating activity of overdose concentrations (30 μmol/l) of TEPA (Fig 4) (TEPA 30, Cu 10). These experiments demonstrated that TEPA and Cu have opposing effects and that it is possible to control long-term proliferation in cultures initiated with CD34 cells by fine modulation of cellular Cu content.

These long-term effects of Cu modulation did not require continuous exposure to either TEPA or Cu. As shown in Fig 6, after 3 weeks no differences between control and treated cultures could be observed. At this time point, TEPA and Cu were washed out and the cells were re-cultured in regular medium (Fig 6, discontinuous

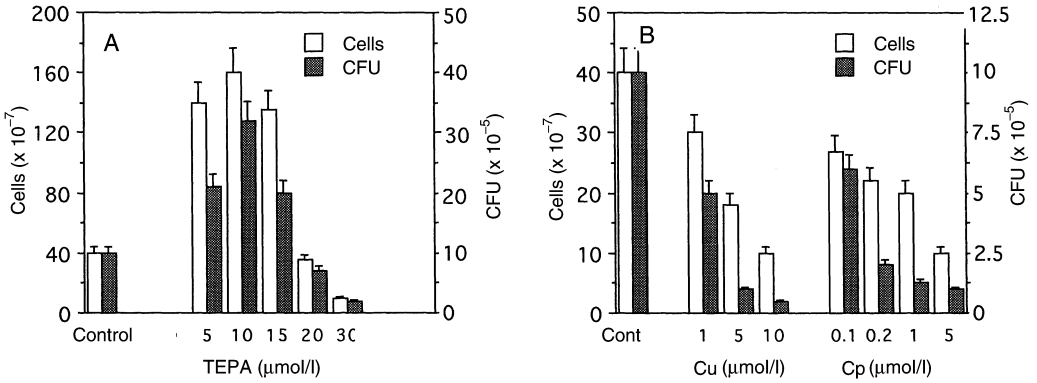

Fig 3. Effect of Cu modulation on long-term cultures. $CD34^+$ cell cultures were continuously supplemented with the indicated concentrations of tetra-ethylenepentamine (TEPA) (A) and Cu sulphate or Cp (B). Number of viable cells and colony-forming unit (CFU) content were determined on week 7 of the culture. Total cells and CFU are presented as accumulative numbers (mean ± SD of four determinations).

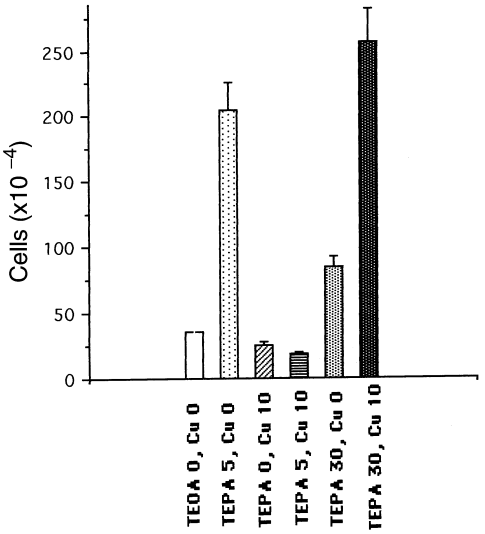

Fig 4. Modulation of long-term cell proliferation by fine-tuning of cellular Cu. $CD34^+$ cell cultures were continuously supplemented with tetra-ethylenepentamine (TEPA) alone, at 5 μmol/l (TEPA 5, Cu 0) or at 30 μmol/l (TEPA 30, Cu 0), with Cu sulphate alone at 10 μmol/l (TEPA 0, Cu 30) or with combinations of both (TEPA 5, Cu 10) (TEPA 30, Cu 10). Control cultures contained neither TEPA nor Cu (TEPA 0, Cu 0). Numbers of cells were determined on week 9 of the culture and are presented as cumulative numbers (mean ± SD of four determinations).

treatment). Determination of total cells and CFU in the long term cultures, weeks 6 and 8, demonstrated that the numbers of total cells and CFU were higher in TEPA-pretreated cultures and lower in Cu-pretreated cultures than in controls. Similar increases in the number of cells and CFU during the long-term cultures was obtained in cultures treated continuously or only for 3 weeks using TEPA.

Indeed, for the long-term effects, Cu modulation was critical during the first week of the $CD34^+$ culture. A significant difference was found in the CFU content of 5-week cultures when TEPA was added to the cultures on d 0 (3×10^4 CFU) or after 6 d (1×10^4 CFU). The results indicated that only when added at the initiation of culture was TEPA able to support long-term CFU expansion.

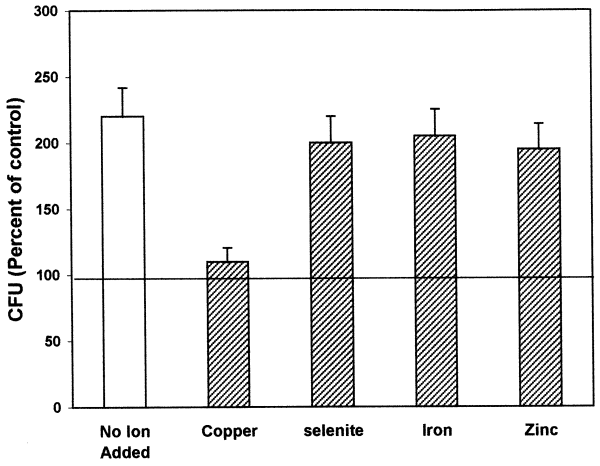

Fig 5. The effect of metal ions on tetra-ethylenepentamine (TEPA) activity. $CD34^+$ cell cultures were supplemented with 15 μmol/l TEPA either with no ion added (except those present in the culture medium) or with the addition of different metal ions; Cu sulphate, sodium selenite, zinc chloride, 5 μmol/l each, and iron-saturated transferrin, 0·3 mg/ml. Colony-forming units (CFU) were determined 4 weeks after culture initiation and are presented as percentage of CFU in control cultures that were supplemented with the respective ions but not TEPA (mean ± SD of four determinations).

Modulation of cellular Cu: effect on cell differentiation

Determination of the lineage-specific antigens CD14 and CD15 on 4-week culture demonstrated that TEPA-treated cultures contained lower percentages of CD14- and CD15-positive cells and Cu-treated cultures contained higher percentages of such cells than control cultures (Fig 7A). In contrast, $CD34^+$ cells in TEPA-treated cultures were twofold higher and in Cu-treated cultures twofold lower than in control cultures (Fig 7C). In accordance with these results, the frequency of CFU (number of CFU/number of viable cells) was high in TEPA-treated cultures and low in Cu-treated cultures compared with control cultures (Fig 7B).

Morphological assessment of 7-week cultures indicated that TEPA-treated cultures contained a high fraction of undifferentiated cells, whereas control cultures contained mainly well-differentiated macrophages (Fig 8).

DISCUSSION

Ions are known to play an important role in basic cell functions such as cell survival, proliferation, differentiation and functionality. Metal ions such as Fe, Ca, Mg and Zn are among those most studied. Relatively little attention, however, has been drawn to the role of Cu. It is well known that both Cu overload and Cu deficiency cause severe pathological conditions owing to multiorgan failure. In the haematopoietic system, Cu deficiency causes pancytopenia (Zidar *et al*, 1977; Hirase *et al*, 1992; Banno *et al*, 1994; Wasa *et al*, 1994). Laboratory studies suggested that the shortage of functional blood cells is owing to a block in development of the haematopoietic stem and progenitor cells (HPC) in a Cu-deficient microenvironment. HPC were present in the patients and developed normally in culture supplemented with normal serum, but failed to develop in autologous, Cu-deficient-serum (Zidar *et al*, 1977).

Further insight into the role of Cu came from studies using established cell lines. Bae & Percival (1994) have shown that induction by retinoic acid of HL-60, a human myeloblastic leukaemic cell line, into mature neutrophils was associated with accelerated uptake of Cu during the early stages of differentiation, but not during the late stages. Eventually, the cells accumulated twice the amount of Cu per cellular protein content as uninduced cells. Addition of excess Cu to regular serum-supplemented culture medium modestly increased retinoic acid-induced differentiation (Bae & Percival, 1993). Recently, we have shown that cerulo-

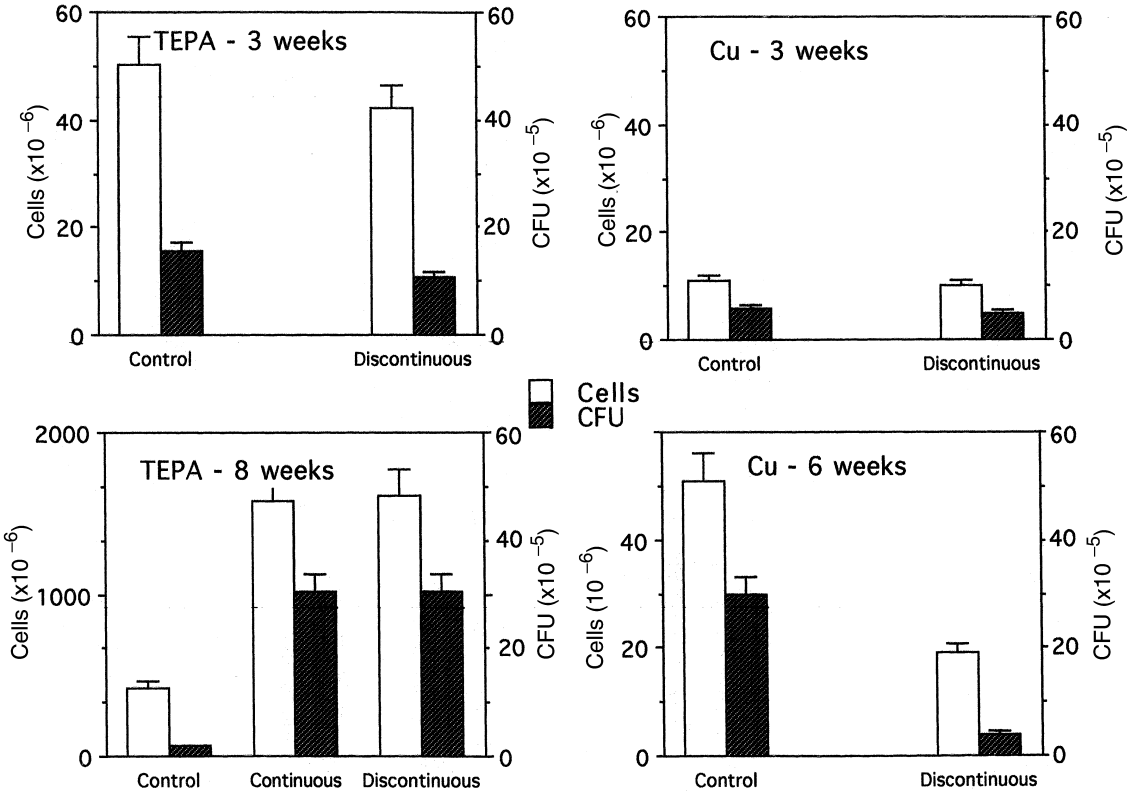

Fig 6. Effect of duration of Cu modulation on the long-term culture proliferative potential. Cu modulation was accomplished by treatment of $CD34^+$ cells for 3 weeks with or without (control) tetra-ethylenepentamine (TEPA) (15 μmol/l) or Cu (10 μmol/l) (top panels). The cultures were then washed and split (bottom panels): one culture continued to grow in the presence of the modulator (TEPA or Cu respectively) (continuous), while the other was recultured in modulator-free medium (discontinuous). The cultures were terminated after 6 weeks (Cu-treated) or 8 weeks (TEPA-treated). Total cells and CFU cumulative numbers at week 3 and after termination of the cultures are presented.

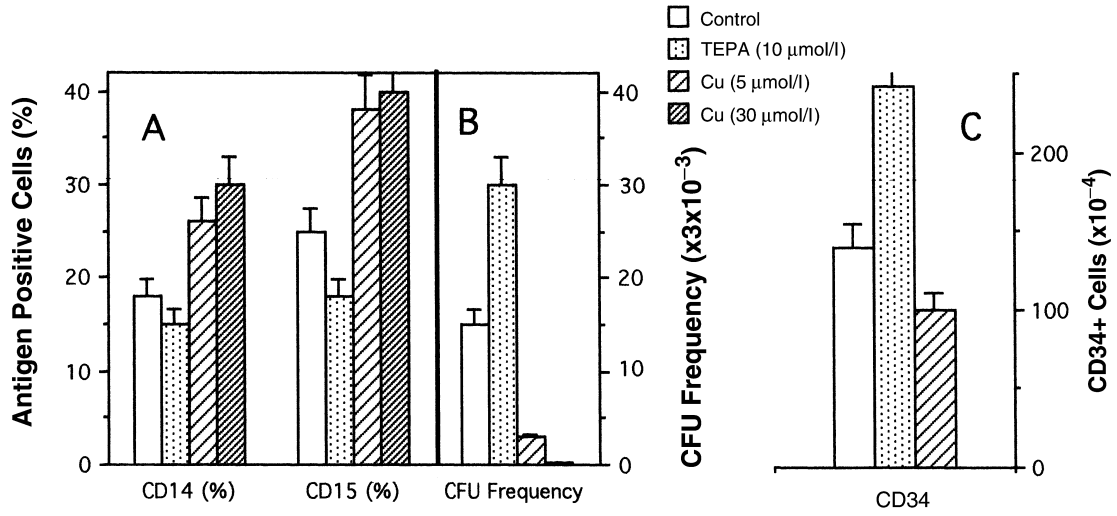

Fig 7. Effect of Cu modulation on cell differentiation. $CD34^+$ cell cultures were treated for 4 weeks with tetra-ethylenepentamine (TEPA) (10 μmol/l), Cu sulphate (5 or 30 μmol/l) or none (control). The percentage of CD14, CD15-positive cells (A) and colony-forming unit (CFU) frequency (number of colonies/number of viable cells) (B) were determined after 4 weeks. The numbers of $CD34^+$ (C) were determined after 3 weeks, and are presented as cumulative numbers per ml culture.

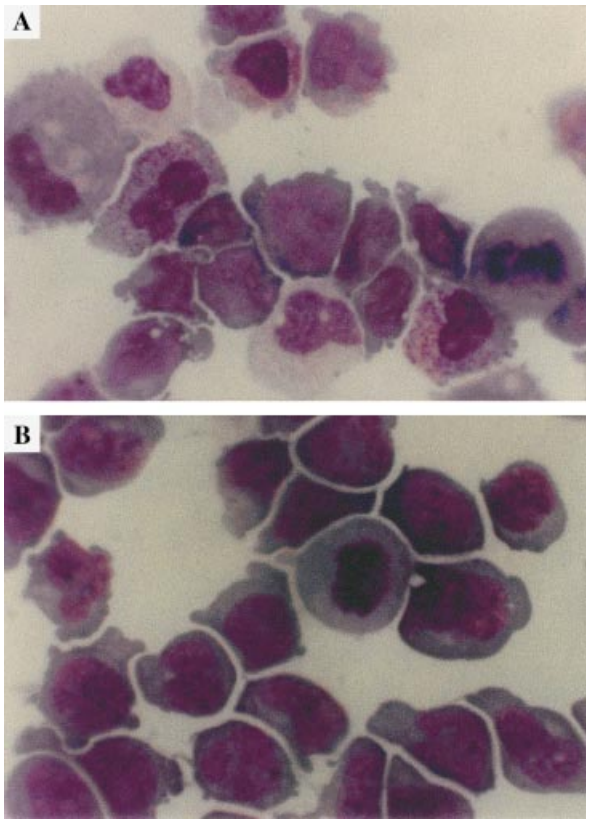

Fig 8. Cell morphology of tetra-ethylenepentamine (TEPA)-treated and untreated cultures. Giemsa-stained photomicrographs (original magnification ×2190) of cells derived from 7 week cultures supplemented without (A) or with (B) TEPA (15 μmol/l). The population in A contained 93 ± 5% cells with blast morphology whereas in B there were only 6 ± 3% blast cells.

plasmin (CP), the main serum Cu-binding protein, is, by itself, a strong inducer of HL-60 cell differentiation (Peled *et al*, 1998). In this context, it is interesting to note that Cp has been shown to have a therapeutic effect in patients with aplastic anaemia (Shimizu, 1979).

TEPA is a synthetic linear polyamine that chelates Cu and, when added to cell cultures, decreased cellular Cu content (Percival & Layden-Patrice, 1992). We have recently found that TEPA inhibited erythroid differentiation induced in the murine erythroleukaemia cells as well as myeloid differentiation induced by retinoic acid in HL-60 and other myeloid leukaemic cells (unpublished observations).

In the present study, we investigated the involvement of cellular Cu in the regulation of the proliferation and differentiation of HPC. For this purpose, we used cultures of cord blood-derived purified CD34 cells grown in serum and cytokine-supplemented liquid medium. Under the conditions used in the present study, HPC expansion continued for 6–8 weeks, and 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. This system serves as a model for studying haematopoietic and other organs' stem cell biology and, in addition, may provide a valuable source of *ex vivo* expanded cells for haematopoietic transplantation.

The culture medium used in the present study contained 17 ng/ml Cu. After 2 d in this medium, the CD34 cells contained 7 ng Cu per 10^7 cells. Intracellular Cu content could be modulated, either decreased by addition of TEPA or increased by addition of Cp or Cu sulphate to the culture medium. Extreme Cu contents, either low (following addition of > 30 μmol/l TEPA) or high (> 30 μmol/l Cu), were incompatible with cell proliferation. Optimal modulation could be achieved by using either compound at concentrations within the non-toxic range or by fine-tuning by adding together defined doses of both TEPA and Cu (or Cp). In both cases, it is the cellular Cu content that determines the result (toxicity, inhibition or enhancement of differentiation or proliferation).

As demonstrated by CFU frequency (number of colonies/number of viable cells), increased Cu content was associated with accelerated differentiation resulting in a shorter period of proliferation, while decreased Cu was associated with delayed differentiation, associated with prolonged period of proliferation. These resulted in higher expansion of undifferentiated cells in TEPA-treated cultures and lower expansion in Cu-treated cultures than in controls. This effect of TEPA was mediated through changes in the cellular Cu content, as Cu but not other transition metal ions reverse it.

The biological effects of Cu modulation were not discernable during the first 3 weeks, but became apparent later. Yet, these long-term effects of Cu modulation did not require continuous exposure to either TEPA or Cu. When Cu was modulated (by adding TEPA or Cu to the cultures) for only 3 weeks, the long-term effects were similar to those in cultures in which Cu was modulated continuously.

Although the effects on proliferation and differentiation were correlated with total cellular Cu content, they might involve a specific subcellular site or component (which is affected by the total cellular Cu content) that directly affects a specific step in cell differentiation. Thus, Cu through cellular redox potential has been shown to affect transcription factors (Hainaut *et al*, 1995; Kudrin, 2000; Vanacore *et al*, 2000).

Although both the proliferation and differentiation processes are affected (in a reciprocal manner) by the Cu content, we hypothesize that differentiation is the primary target, while the effect on proliferation is a secondary consequence. This notion is supported by our findings that short-term Cu modulation was sufficient to cause the long-term effects, suggesting that Cu regulates a decision process for differentiation taken early during the culture. Recent preliminary results indicated that TEPA delays, within 3–6 d, transition of an early subset of most primitive stem cells with the $CD34^+CD38^-lin^-$ phenotype into more mature $CD34^-CD38^+lin^+$ cells.

Ex vivo expansion of pluripotent stem cells and lineage-specific progenitor cells may have important clinical application in cell therapy. These newly discovered effects of Cu modulation by Cu or Cu chelators might be used for

maximizing the *ex vivo* expansion of haematopoietic and other tissues' stem and progenitor cells.

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