Copper Is Required to Maintain Cu/Zn-Superoxide Dismutase Activity During HL-60 Cell Differentiation (43576)

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Abstract. **The objective of these studies was to characterize the relationship between copper levels and Cu/Zn superoxide dismutase (Cu/Zn-SOD) during cellular differentiation. It was hypothesized that the decrease in Cu/Zn-SOD activity that accompanied differentiation would be reversed by supplementing the culture medium with copper. HL-60 cells, a human promyelocytic cell line, were induced to differentiate with retinoic acid and were concurrently supplemented with copper or a copper chelator, tetraethylenepentamine. The results showed that retinoic acid-treated cells contained more copper after differentiation. When the medium was supplemented with copper during retinoic acid treatment, the differentiating cells accumulated more copper than the nondifferentiating cells. Differentiation was accompanied by a significant reduction in Cu/Zn-SOD activity and a slight reduction in Cu/Zn-SOD protein. Activity returned to control values when an extracellular source of copper was provided. Incubation of retinoic acid-treated cells with the chelator showed that they lost proportionally less copper than the noninduced controls. Levels of Cu/Zn-SOD protein were not affected by the copper or chelator treatments. It was concluded that the requirement of differentiating HL-60 cells for copper is not related to providing copper for Cu/Zn-SOD activity. If a supplemental source is not supplied in the medium, then the cells may acquire copper from an intracellular source, namely Cu/Zn-SOD.** [P.S.E.B.M. 1993, Vol 203]

Copper has been shown to be an essential trace element for proper functioning of the immune system (1, 2). A copper deficiency will result in alterations in the acute phase response, T lymphocyte functioning, and B lymphocyte antibody response. Another sign of copper deficiency in humans is neutropenia (3, 4). Neutrophils are the first line of defense against infection, and they protect the host by engulfing and killing invading microorganisms. The specific function of copper in the neutrophil is not known. Early work by others suggested that copper deficiency appeared to arrest the maturation of human bone marrow white cells (5).

Phagocytosis by the neutrophil initiates a burst of oxygen consumption, also known as the respiratory burst, which results in the production of superoxide anion, hydrogen peroxide, singlet oxygen, and hydroxyl radicals (6, 7). These active oxygen species are potentially damaging to the cell membrane and organelles; therefore, it seems necessary for the cell to have mechanisms available to protect itself. One such protective mechanism is Cu/Zn-superoxide dismutase (Cu/Zn-SOD), which is able to dismutate the superoxide anion to produce oxygen and hydrogen peroxide (8).

Contrary to what one would expect, Speier and Newburger (9) found that as myeloid cells develop the ability to initiate the respiratory burst, SOD activity declined. Their study showed that when HL-60 cells differentiate toward neutrophils, the activities of both the mangano and the copper-zinc enzymes were reduced. However, an extracellular source of copper was not provided to the cells. We hypothesized that preservation of Cu/Zn-SOD activity is important to the cell, but it can only be maintained during differentiation when a source of copper is provided. To determine this, we examined Cu/Zn-SOD activity in differentiating HL-60 cells incubated with copper or a metal chelator. Our results show that the specific activity of Cu/Zn-SOD was not maintained during differentiation unless

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the medium was supplemented with copper. Although the role(s) for copper in neutrophil development has not been fully explored, a copper dependency for Cu/Zn-SOD activity and its effect on cellular protection could explain the basis by which copper deficiency results in neutropenia.

Materials and Methods

Cell Culture. HL-60 cells were purchased from the American Type Culture Collection (Rockville, MD) at passage 11 and maintained in RPMI medium containing HEPES buffer (Cellgro; Mediatech, Inc., Herndon, VA), 10% fetal bovine serum (ICN-Flow, Costa Mesa, CA), 2 m*M* glutamine (Mediatech), and antibiotics. The complete medium contained 0.5 μM copper; this is about 10-fold less than physiological serum levels (see below). The chelator tetraethylenepentamine (TEPA) was purchased from Sigma (St. Louis, MO), dissolved in phosphate-buffered saline and sterile-filtered before addition to the cells in culture. Cells were maintained at a concentration between 2.5–10 $\times$ 10^8/liter, and experiments were initiated at a concentration of 2.5 $\times$ 10^8 cells/liter. Cells were used for experiments up to passage 40. A stock solution of retinoic acid (RA; 1 m*M*) was prepared fresh in 95% ethanol for each experiment. A final concentration of 1 μM was added to culture flasks (10), such that the concentration of ethanol was less than 0.1%. Copper (certified copper reference solution, free metal in 30 g/liter nitric acid; Fisher Chemical, Fair Lawn, NJ) was added to cell cultures at concentrations ranging from 8 to 48 μM. Estimates of serum levels of copper range from 8 to 15 μM under normal physiological conditions. Levels of serum copper can increase 300% during periods of stress or inflammation. TEPA was added at a concentration of 50 μM (11). Neither copper nor TEPA supplementation had any effect on the growth or viability of the cells.

Copper Determination. After washing the cells with phosphate-buffered saline, an aliquot of 2 $\times$ 10^6 cells was pelleted in a metal-free Eppendorf tube. The cell pellet was adjusted to a concentration of 1 $\times$ 10^{10}/liter with 30 m*M* ultrapure nitric acid (Optima, FisherBrand). Samples were sonicated for 1 min on ice, and copper was determined at a wavelength of 324.7 in a graphite furnace atomic absorption spectrophotometry. The following times and temperatures were used: drying at 95°C for 45 sec with 15-sec ramp, charring at 900°C for 30 sec with 10-sec ramp, and atomization at 2300°C for 10 s. The peak area was integrated for 10 sec.

Cu/Zn-SOD Activity. The SOD assay was performed in flexible microtiter plates using pyrogallol (1,2,3-benezenetriol; Sigma), as originally described by Marklund and Marklund (12) and modified by Prohaska *et al.* (2). Cell homogenates were extracted with 0.4 vol of chloroform and ethanol (15:25, $CHCl_3$:EtOH) to precipitate the manganese SOD (2) and then centrifuged at 14,000*g* for 4 min before aliquoting to microtiter plates. Fifty microliters of 2 $\times$ 10^7 cells/ml were serially diluted (1:1) in triplicate with 50 m*M* Tris buffer containing 1 m*M* diethylenetriaminepentaacetic acid, pH 8.2. Four dilutions were routinely implemented. Each well received an additional 50 μl of Tris buffer. Before reading the plates, sodium azide (10 m*M*) was added to each well to inhibit a cellular component that enhanced the oxidation of pyrogallol (13; S. S. Percival, submitted). Lastly, 50 μl of 0.2 m*M* pyrogallol in 10 m*M* HCl containing 1 m*M* diethylenetriaminepentaacetic acid were added. Absorbance at 340 nm was monitored at room temperature every 10 sec in shaker mode in a kinetic microplate reader (UVMax; Molecular Devices, Menlo Park, CA) for 3 min.

The program software (Softmax; Molecular Devices) calculates the change in absorbance as mOD/min. Each sample was analyzed in triplicate with four serial dilutions and had a matched control containing no cell homogenate in order to determine the autoxidation rate of pyrogallol. The percent inhibition of pyrogallol autoxidation by cellular Cu/Zn-SOD was calculated for the average of each dilution using the corresponding dilution of control solution without cell sample. This was calculated from the equation: $100 \times (mOD/min_A - mOD/min_S)/mOD/min_A$, where A is the maximum velocity of the autoxidation without cell homogenate, and S is the maximum velocity of the oxidation with cell homogenate. One unit of activity is defined as that amount of enzyme that inhibits the autoxidation of pyrogallol by 50%. The percent inhibition was plotted against the volume of supernatant (four dilution values), and the units of activity were calculated from the linear portion of the curve when $y = 50\%$.

Cu/Zn-SOD Enzyme-Linked Immunosorbent Assay. Cu/Zn-SOD protein was quantitated by enzyme-linked immunosorbent assay (ELISA). Rabbit antihuman Cu/Zn-SOD antibodies were prepared by injecting rabbits with human erythrocyte superoxide dismutase supplied by Sigma. The rabbit antiserum was used without further purification. The Western blot procedure determined the specificity of the antibody. One protein band was detected that corresponded to a mol wt of 16,000, the mol wt of the monomer of Cu/Zn-SOD protein. HL-60 cells were prepared for the ELISA by washing, sonicating, and extracting with chloroform and ethanol at a ratio of 15:25 (2). The ELISA was prepared by coating wells of a 96-well microtiter plate (Corning, Medfield, MA) with 100 μl of chloroform:ethanol-extracted human erythrocyte Cu/Zn-SOD (Sigma) in deionized distilled water (MilliQ system) or with 100 μl of chloroform:ethanol-extracted cell cytosol. Cu/Zn-SOD standards were di-

luted from 500 pg to 7.8 pg with phosphate-buffered saline. After incubating the plate overnight, rabbit serum containing anti-Cu/Zn-SOD antibodies was diluted 1/1000 in 5 g/liter nonfat dry milk, and 100 μl were aliquoted to each well. After washing, goat anti-rabbit IgG (100 μl) conjugated with horseradish peroxidase (Bio-Rad Laboratories, Richmond, CA) diluted 1/1000 in 5 g/liter nonfat dry milk was added to each well. After incubation at 22°C for 2 hr, this solution was decanted, and the plate was washed. *o*-Phenylenediamine (0.67 g/liter; Dako Corp., Carpenteria, CA) in 0.1 *M* citric acid/phosphate buffer, pH 5.0, containing 0.25 ml/liter H_2O_2 was then added to each well. After 15 min of incubation in the dark, the reaction was stopped with 100 μl of 1 *M* H_2SO_4, and the absorbance at 490 nm was determined (UVMax, Molecular Devices).

Statistical Analyses. Statistical evaluation of the data was conducted by analysis of variance using the software program Instat (version 1.14) produced by GraphPad Software (San Diego, CA). Significantly different means were detected by a post-hoc *t* test, using an uncorrected *P*-value less than 0.05. Analyses were performed in triplicate with at least three different flasks (see figure and table legends).

Results

Differentiation of HL-60 by RA treatment was established by microscopic examination after differential staining, by nitroblue tetrazolium reduction assay (10), and by production of superoxide anion after acute phorbol ester stimulation (14) (data not shown).

Cu/Zn-SOD activity declined about 50% in HL-60 cells during the 4 days of RA treatment (Fig. 1). As these cells differentiated along the granulocytic pathway, the activity of Cu/Zn-SOD was reduced.

In contrast to Cu/Zn-SOD activity, cell-associated copper increased during the course of differentiation (Fig. 2). By the end of the 4 days of RA treatment, the cells had about 20–30% more copper than the cells not induced to differentiate.

To examine the extent of copper accumulation in the RA-induced cells, several levels of copper were added to the medium of HL-60 cells during RA treatment. All cells accumulated copper from the medium, but the RA-induced cells had a much more pronounced accumulation (Fig. 3). Nontreated HL-60 cells accumulated 3.8-fold more copper at a medium copper concentration of 48 μM than cells incubated in nonsupplemented medium, which contained 0.5 μM copper. The RA-induced cells incubated with 48 μM copper accumulated 6-fold more copper than RA cells in the nonsupplemented medium.

Cellular copper status was further investigated by examining the specific activity of Cu/Zn-SOD in the cells under varying conditions (Table I). Cu/Zn-SOD protein, as determined by the ELISA procedure, was

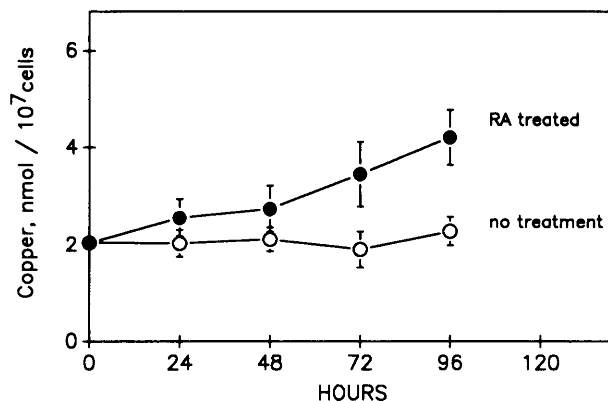

Figure 2. Copper content increased during differentiation of HL-60 cells. HL-60 cells (2.5 × 10^{10}/liter) were incubated alone or with 1 μM RA. Flasks were harvested every 24 hr. Data points are the mean ± SD (*n* = 3 flasks).

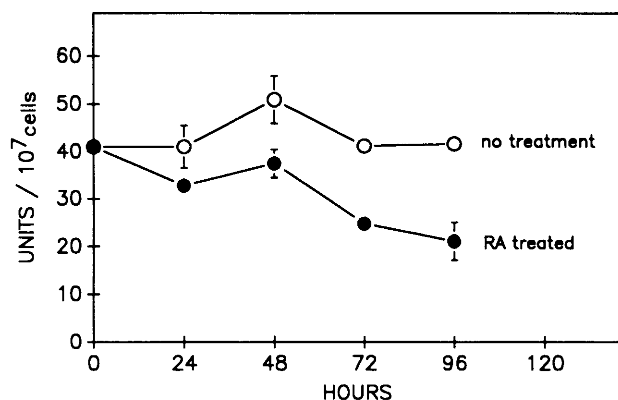

Figure 1. Cu/Zn-SOD activity declined during differentiation of HL-60 cells. HL-60 cells (2.5 × 10^{10}/liter) were incubated alone or with 1 μM RA. Flasks were harvested every 24 hr. One unit of Cu/Zn-SOD activity is defined as the amount of enzyme that will inhibit the autoxidation of pyrogallol by 50%. Data points are the mean ± SD (*n* = 3 flasks).

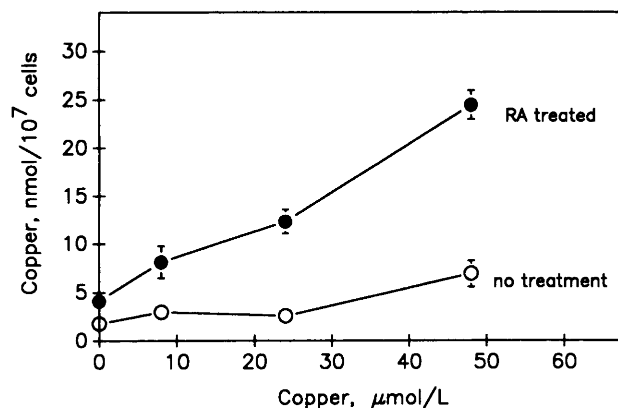

Figure 3. Differentiating HL-60 cells accumulated more copper than noninduced cells. HL-60 cells (2.5 × 10^{10}/liter) were incubated alone or with 1 μM retinoic acid. Various levels of copper were added at time 0, and flasks were harvested at the end of 96 hr of incubation. Data points are the mean ± SD (*n* = 3 flasks).

Table I. Measures of Copper Status in HL-60 Cells and Retinoic Acid-Treated HL-60 Cells[a]

	Copper (nmol/10^7 cells)	Cu/Zn-SOD activity (units/10^7 cells)	Cu/Zn-SOD protein (pmol/10^7 cells)	Sp act (units/pmol protein)
HL-60	3.29 ± 0.20‡	59.9 ± 5.0†	10.0 ± 2.9*	6.0
HL + Cu	4.49 ± 0.23†	78.8 ± 11.5*	9.6 ± 2.4*	8.2
HL + TEPA	2.02 ± 0.25§	18.3 ± 7.6‡,§	10.2 ± 1.8*	1.8
RA Treated	4.25 ± 0.26†	26.6 ± 2.3‡	6.3 ± 1.3†	4.2
RA + Cu	11.69 ± 0.95*	62.9 ± 6.8†	7.7 ± 1.6*,†	8.2
RA + TEPA	3.24 ± 0.52‡	13.6 ± 5.1§	7.1 ± 1.3*,†	1.9

[a] HL-60 cells at a beginning concentration of 2.5×10^8/liter were incubated with 1 μmol/liter of retinoic acid for 4 days. Copper (12 μmol/liter) or TEPA (50 μmol/liter) was added at Time 0. Data are expressed as means ± SD; $n = 4$ for Cu/Zn-SOD and $n = 6$ for copper levels. Values in a column not sharing a common symbol (*, †, ‡, §) are significantly different at $P < 0.05$. One unit of activity is defined as that amount of enzyme that inhibits the autoxidation of pyrogallol by 50%.

about 40% less in the RA-induced cells than in the noninduced cells. Cu/Zn-SOD activity, however, was 55% less in RA-induced cells than in noninduced cells. Specific activity was 30% less in the RA-induced cells than in the noninduced cells, largely due to a loss in activity of the enzyme.

To determine the effect of copper availability on copper levels and Cu/Zn-SOD during differentiation, HL-60 cells were incubated with copper-supplemented medium. Copper supplementation resulted in a third more copper associated with the control HL-60 cell, while the RA-induced cell contained nearly 3-fold more copper (Table I). Copper supplementation also resulted in an increase in Cu/Zn-SOD activity, but had no effect on the level of Cu/Zn-SOD protein. Supplemental copper produced the highest Cu/Zn-SOD specific activity, and this effect was not influenced by differentiation.

When these cells were incubated with a high affinity copper chelator, both copper levels and Cu/Zn-SOD activity were reduced (Table I). The chelator reduced copper levels by about 40% in the control HL-60 cells, but only reduced copper levels in the RA-induced cells by 25%. The absolute amount of copper lost was greater in the noninduced cells (1.3 nmol/10^7 cells) than in the RA-induced cells (1.0 nmol/10^7 cells). Cu/Zn-SOD activity was markedly reduced after incubation with the chelator. Noninduced cells treated with TEPA lost 70% of the activity, and RA-induced cells lost 50% of the activity compared to their respective nonchelated control cells. The chelator had no effect on the level of Cu/Zn-SOD protein.

Discussion

In humans, copper deficiency results in neutropenia (3). Examination of a bone marrow aspirate from a copper-deficient person suggested that copper deficiency arrests maturation of neutrophils in the bone marrow (5). To investigate the cause of neutropenia related to copper deficiency, we examined copper metabolism and the relationship of Cu/Zn-SOD levels to copper levels in a cell capable of differentiating along the granulocytic lineage (10, 15). Aspects of copper metabolism were characterized during induced differentiation of HL-60 cells. Retinoic acid treatment resulted in cells that contained more copper after differentiation and accumulated more copper, but had reduced Cu/Zn-SOD activity. Apparently, the additional copper in the RA-induced cell was not sufficient to sustain Cu/Zn-SOD activity. Providing an extracellular source of copper maintained the activity of the enzyme in the RA-induced cells at the level found in HL-60 cells.

HL-60 cells have been shown to be marginally copper deficient after incubation with 50 μM TEPA (11). When the cells were incubated with the copper chelator TEPA during differentiation, the RA-induced cell retained more copper than the noninduced cells incubated with TEPA. Both cell types lost a greater proportion of their Cu/Zn-SOD activity than of their copper, suggesting that a significant proportion of chelated copper was derived from Cu/Zn-SOD. TEPA-treated cells, however, still differentiated and acquired the respiratory burst mechanism to the same extent as the control RA-treated cells (11).

These data suggest that there is an obligatory role for copper in HL-60 cell differentiation that is not related to providing the catalytic cofactor for Cu/Zn-SOD. Exactly what those functions are is unknown at this time. The HL-60 cell loses mitochondria upon differentiation (16); therefore, it would be unlikely that cytochrome *c* oxidase would require a substantial portion of that copper. Further experimentation is necessary to determine what the RA-induced cell does with the copper and why it has more copper after differentiation.

Differentiation has been shown to be accompanied by an increase in SOD levels in other cell types (16, 17), yet the RA-treated HL-60 cell showed less Cu/Zn-SOD activity than the nondifferentiated cell. Beckman *et al.* (18) showed that Friend erythroleukemic cells would differentiate when treated with liposomes containing Cu/Zn-SOD. However, they also reported that

differentiation did not occur when cells were incubated with other antioxidants, but was enhanced when incubated with oxidative compounds. Differentiation appeared to prefer pro-oxidation, yet Cu/Zn-SOD is known to function as an antioxidant. This paradox can be explained if copper dissociated from Cu/Zn-SOD for the differentiation process. If this hypothesis is true, then supplemental copper would favor differentiation, and we have evidence that this occurs (B. Bae, and S. S. Percival, unpublished data). The differentiated RA cells may have less Cu/Zn-SOD activity because copper is used in the obligatory differentiation process. Therefore, neutropenia as a result of copper deficiency does not appear to be correlated with the loss of protection by Cu/Zn-SOD. The HL-60 cells were able to differentiate at a time when Cu/Zn-SOD activity was decreasing. Differentiation results in a decrease in some proteins, such as myeloperoxidase, but acid phosphatase, lysozyme, and some plasma membrane receptors are increased (15). Thus, the decrease in Cu/Zn-SOD protein is not a general phenomenon of differentiating HL-60 cells, but neither is it unique. The maintenance of Cu/Zn-SOD activity is important to the cell, but not as important as it is for this hypothesized differentiation function. Cu/Zn-SOD activity can be maintained when an additional amount of copper is provided.

These experiments also show that changes in copper levels are paralleled by changes in Cu/Zn-SOD activity, supporting the idea that the enzyme activity is a good candidate for use as a measure of copper status. Erythrocyte Cu/Zn-SOD activity has been used by many researchers for this purpose (19–21). Recently, Babu and Failla measured several parameters of copper metabolism in rat neutrophils (14). They found that Cu/Zn-SOD activity was very sensitive to changes in dietary copper, and while other functions were also influenced, Cu/Zn-SOD activity was the first to decline under deficient conditions and the first to be restored upon repletion of copper. The neutrophil has a very rapid half-life of 2 days or less, which favors neutrophil Cu/Zn-SOD activity as a candidate for a measure of copper status. We have shown that the Cu/Zn-SOD activity of an immature neutrophil-like cell is, in fact, readily influenced by copper, which supports the utility of this enzyme as a measure of copper status.

In summary, the differentiated HL-60 cell (RA) was able to accumulate copper in both a time- and concentration-dependent manner. The differentiated cell was characterized by lower Cu/Zn-SOD protein and activity. When an extracellular source of copper was provided, the cell was able to maintain Cu/Zn-SOD activity levels comparable to those in the nondifferentiated control cell. The ability to manipulate Cu/Zn-SOD activity as well as the fact that the neutrophil has a short half-life implicate the activity of granulocyte Cu/Zn-SOD as a practical measure of copper status. The data suggest that Cu/Zn-SOD is not as important for cellular protection during differentiation, and that neutropenia, or lack of cell differentiation, may require copper for some other as yet unknown function.

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