Copper uptake and intracellular distribution during retinoic acid-induced differentiation of HL-60 cells

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Because neutropenia is a sign of copper deficiency, the HL-60 cell line was used to examine copper distribution during cellular differentiation. This study measured the accumulation and distribution of copper in differentiating (retinoic acid-treated) and nondifferentiating (untreated) HL-60 cells by radiolabeling the cells with ^{67}Cu. The intracellular distribution of total copper as well as ^{67}Cu was examined. Retinoic acid-treated cells had twice as much copper as control cells. Copper uptake from ^{67}Cu labeled ceruloplasmin was greater in retinoic acid-treated cells at 24 and 48 hr after induction compared with the control cells. The intracellular distribution of ^{67}Cu was found predominantly in high molecular weight fractions (100,000 D) and a low molecular weight fraction (20,000 D). The high molecular weight protein had greater ^{67}Cu associated with it in the retinoic acid-treated cells than in the untreated control cells. This protein may be responsible for the greater levels of copper found in the retinoic acid treated cells. Copper uptake is clearly regulated during the process of differentiation.
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Introduction

Copper is an essential trace element for cellular functions. It is a required cofactor for cytochrome c oxidase and the copper-zinc form of superoxide dismutase (Cu/Zn-SOD), which are found ubiquitously, and for tyrosinase, dopamine β-hydroxylase and lysyl oxidase, which are located in more specialized tissues. However, not all cellular copper is accounted for by these enzymes. Clearly, other roles for copper exist that have yet to be discovered.

One of the signs of copper deficiency is neutropenia.[1,2] Neutropenia in association with copper deficiency has been suggested to result from the abnormal development of myeloid cells,[3,4] suggesting copper plays a role in the differentiation process.

Previous results in this lab have supported a role for copper in the development of the neutrophil using retinoic acid (RA) differentiation of HL-60 cells as the model.[5] Differentiation by RA results in a cell that is granulocytic and contains more copper than the undifferentiated cell. The RA-treated cell, however, has less Cu/Zn-SOD activity, which suggests that the copper that is present in greater quantity is not utilized for the activity of Cu/Zn-SOD.[5,6] In fact, RA-treated cells require supplemental copper to keep the specific activity of Cu/Zn-SOD at control noninduced levels.[6] Copper accumulation may result from an enhanced uptake and/or a decreased efflux. The purpose of these studies was to measure the accumulation and distribution of copper in differentiating (RA-treated) and nondifferentiating (untreated) HL-60 cells. The intracellular distribution of total copper and radioactive ^{67}Cu as well as the rate of ^{67}Cu uptake in RA-treated and untreated cells was examined.

Methods and materials

Cell culture

Human promyelocyte leukemic HL-60 cells were maintained in complete RPMI 1640 medium containing 10% fetal bovine serum, 2 mmol/L glutamine, and antibiotics at 37° C in a 5% CO_2, 95% air atmosphere. Cells were induced to differentiate toward granulocyte with 1 μmol/L retinoic acid prepared in 95% ethanol such that the final concentration of ethanol was less than 0.1%.[7] After 4 days of incubation with retinoic acid, more than 85% of the promyelocyte

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cells were differentiated beyond the myelocyte stage, as estimated by counting cells stained with a modified Wright's stain and determination of the morphological characteristics of the various stages of neutrophil development.[5] Uptake of ^{67}Cu from ^{67}Cu-labeled ceruloplasmin (^{67}Cu-Cp) was determined every 24 hr after initiating RA treatment. Total copper levels and total copper distribution as well as ^{67}Cu distribution was examined in RA-treated and untreated HL-60 cells.

^{67}Cu labeling of ceruloplasmin

Ceruloplasmin (Cp) labeling was performed by a previously described method.[8] Lyophilized Cp (Vital Products, St. Louis, MO USA) was dissolved in 0.05 mol/L HEPES buffer, pH 7.2, at a concentration of 10 g/L. Ascorbic acid was added to a final concentration of 0.05 g/L to catalyze the exchange of Cu molecules in Cp with radioactive ^{67}Cu. Carrier-free ^{67}Cu in 0.1 mol/L HCl (Los Alamos National Labs, Los Alamos, NM USA) was neutralized by 1 N NaOH and then 200 μCi was added to 1.0 mg of Cp. The mixture was allowed to react for 1 hr at 25° C and then eluted through a Sephadex G-50 column (33 × 1 cm) previously equilibrated with 0.05 mol/L HEPES containing 1.0 g/L bovine serum albumin (BSA) and washed with 0.05 mol/L HEPES. A 1.0 mL fraction containing 1.0 mg of ^{67}Cu-Cp was collected. This procedure results in incorporation of all radioactivity into the void volume fractions and recovery of Cp's absorbance ratio at 610 and 280 nm after radiolabeling.

Copper uptake studies

Cellular copper accumulation was determined from 1 to 30 min in both untreated and RA-treated cells with RA present in the incubation buffer. Cells were harvested, washed three times with phosphate buffered saline (PBS), and resuspended in PBS containing 30 g/L BSA and 1.0 μmol/L retinoic acid at a concentration of 1×10^{10} cells/L. ^{67}Cu-Cp (2.0 μmol/L) was added to the cell suspension that was equilibrated to either 37°C or 4°C. At indicated times, 10^6 cells were removed to ice-cold PBS and washed three times with 0.5 mL of ice-cold PBS. Cell pellets were transferred to 20 mL scintillation vials, and the radioactivity was measured in a liquid scintillation counter (LSC 6000SC Beckman, Fullerton, CA, USA). Net uptake was determined by subtracting the cell-associated activity of ^{67}Cu determined at 4° C from that at 37° C. Replicate experiments were performed six times at each time point.

Gel filtration chromatography

Intracellular distribution of copper was studied by gel filtration chromatography. Molecular weight markers included Cp (Vital Products), Cu/Zn-SOD (human erythrocyte, Sigma Chemical Co. St Louis, MO USA), and lysozyme. Control cells or RA-treated (96 hr) cells were incubated for 3 hr with 2 μmol/L ^{67}Cu-Cp at 37° C. A total of 2×10^7 cells were ruptured by sonication in 0.05 mol/L HEPES buffer, pH 7.2 containing protease inhibitors (0.1 mmol/L phenylmethylsulfonyl fluoride, 20 μmol/L leupeptin, and 0.7 mg/L pepstatin) and ultracentrifuged at 600,000*g* for 20 min at 4° C (Beckman Optima TL Ultracentrifuge, TLA 100.4 fixed angle rotor). The supernatant was fractionated on a Superose 12B column (Pharmacia, Piscataway, NJ USA) equilibrated with 0.05 mol/L HEPES buffer, pH 7.2. Proteins were eluted at a flow rate of 0.5 mL/min into 1 mL fractions and monitored at 280 nm. The ^{67}Cu in each fraction was determined by liquid scintillation counting. Replicate experiments were performed at least four times. Recovery of radioactivity off the column was greater than 90% each time. Recovery of unlabeled (total) copper off the column was approximately 85%.

Analytical methods

To determine total copper, an aliquot containing 2×10^6 cells was transferred and pelleted in a metal-free Eppendorf tube. The pellet was brought to a concentration of 1×10^{10}/L cell equivalents with 0.03 mol/L ultrapure nitric acid (Optima, FisherBrand, Fisher Scientific, Pittsburgh, PA USA). Cells were disrupted by sonication and samples were centrifuged at 14,000*g* for 4 min to remove cellular debris. Cell supernatants were then analyzed by graphite furnace atomic absorption spectrophotometry (GBC Model 902 Double Beam with a System 2000 controller, GBC Scientific Equipment, Inc., Arlington Heights, IL USA) at a wavelength of 324.7 nm, a lamp current of 4.0 mA, and a spectral slit width of 0.5 nm. The following times and temperatures were used: drying at 120° C for a total of 60 sec with a 15 sec ramp; charring at 500° C for 11.5 sec with a 5.5 sec ramp; atomization at 2,300° C for 4.5 sec with a 1.5 sec ramp. Peak area was integrated for 0.3 sec. Fractions from gel chromatography were analyzed without further treatment. Matrix effects in any sample were less than 8% as determined by standard additions to cell samples. Accuracy of the analysis was ensured by analyzing Seronorm (Accurate Chemical and Scientific Corp, Westbury, NY USA), which has a known copper concentration of 1.10 mg/L. Our analysis resulted in an estimated copper value of Seronorm of 1.04 mg/L, with a daily coefficient of variation of less than 6%. Copper analyses was performed on six cultures.

The measurement of Cu/Zn-SOD activity was performed in microtiter plates as previously described[9] and was based on the modification by Prohaska et al.[10] In brief, activity was determined by the inhibition of the rate of the autoxidation of pyrogallol. Cell fractions were extracted with chloroform:ethanol (25:15) to precipitate the manganous-SOD and assayed kinetically in a microtiter plate (UVMax, Molecular Devices, Menlo Park, CA USA) at 340 nm containing a final concentration of 6 mmol/L sodium azide. Fifty percent inhibition of pyrogallol's autoxidation is defined as 1 unit of activity and was determined using four serial dilutions of cell extract, which then were subjected to linear regression analysis.

Statistical analyses

The uptake experiments were analyzed by a two-way analysis of variance (ANOVA) performed by SigmaStat (Jandel Scientific, San Rafael, CA, USA), version 1.01. The two-way ANOVA compared treatment, either untreated or retinoic acid-treated, and the length of time of incubation. Significantly different means were determined by the Student-Newman-Keuls post test of multiple comparisons, and significant differences were detected at $P < 0.05$.

Results

The distribution of copper in whole cells and in the supernatant after ultracentrifugation of RA-treated and HL-60 cells is shown in *Table 1*. RA-treated cells have significantly more copper (50% more) than the untreated cells but about a third less cellular protein. When cells are disrupted by sonication and ultracentrifuged, the amount of copper per cell (equivalent to cell number) is not significantly different in RA-treated compared with untreated cells, but the protein concentration in RA-treated cells is about half of the untreated cells. Therefore, the supernatant of RA-treated cells have twice the concentration of copper per mg of cellular protein as the untreated control cell supernatant.

Both the untreated cells and RA-treated cells (96 hr of continuous exposure) were incubated with ^{67}Cu-Cp for 3 hr, and the radioactivity associated with the supernatant was measured. The percent of the total added radioactivity found

Table 1 Total copper distribution in HL-60 and retinoic acid-treated HL-60 cells

	Copper	Protein	Copper
	ng/10⁷	*mg/10⁷*	*ng/mg protein*
Whole cell homogenate			
HL-60	12.1 ± 1.2	0.64 ± 0.01	18.9 ± 2.1
RA	18.7 ± 1.4	0.43 ± 0.02	43.5 ± 5.2
Supernatant			
HL-60	10.2 ± 1.3*	0.21 ± 0.01	48.6 ± 5.1
RA	11.2 ± 0.9	0.11 ± 0.01	101.8 ± 9.9

Values are means ± SD of four replicate experiments. Means of HL-60 versus RA-treated are significantly different by an unpaired two-tailed *t* test except where indicated by (*).
RA, Retinoic acid.
Copper was determined by graphite furnace AAS; protein by the Bradford method.
The analyses were performed on cells treated with RA for 96 hr.

in the supernatant of RA-treated cells was 4.27% ± 0.27 compared with 7.15% ± 0.41 in the supernatant of the control cells (expressed in cell equivalents). When expressed on a per mg of cellular protein basis, the percent of radioactivity found in the supernatant was equal between the two cell types.

Copper accumulation was examined by incubating with ^{67}Cu-Cp every 24 hr during the 4 days of RA treatment *(Figure 1)*. Uptake was linear over the duration of the 30 min incubation time with ^{67}Cu-Cp. Furthermore, the addition of retinoic acid itself to the incubation buffer did not significantly affect copper uptake. The rate of uptake was significantly faster 24 and 48 hr after retinoic acid treatment was initiated *(Table 2)*. At 24 hr, a nonlinear biphasic response to ^{67}Cu uptake was observed. Copper uptake was linear for approximately 10 min. Therefore the initial rate of uptake was calculated from these data. The rates of uptake at 72 and 96 hr after retinoic acid was added were not significantly different than control cells at each of the respective times.

The intracellular distribution of ^{67}Cu after incubating cells with ^{67}Cu-Cp is shown in *Figure 2*. Two peaks of radioactivity were observed; a broad peak in fraction 10 and a well-defined peak in fraction 15. These fractions correspond to molecular weights of approximately 100,000 and 20,000 daltons, respectively. Cu/Zn-SOD, used as a molecular weight marker for column calibration, was located in fraction 13, distinct from either of the radioactive ^{67}Cu peaks. The activity of Cu/Zn-SOD was also located in fraction 13. Untreated control cells had a greater proportion of ^{67}Cu in fraction 15 than in fraction 10. The RA-treated cells had roughly the same proportion of radioactivity in fractions 10 and 15.

The intracellular distribution of total copper is shown in *Figure 3*. The majority of copper was located in fraction 13, the same fraction where Cu/Zn-SOD activity is found. Copper level was greater in fractions 9 and 10 of the RA-treated cell supernatant than in fractions 9 and 10 of the untreated cells, but other fractions of the RA-treated cells had less copper than the corresponding untreated cell fractions.

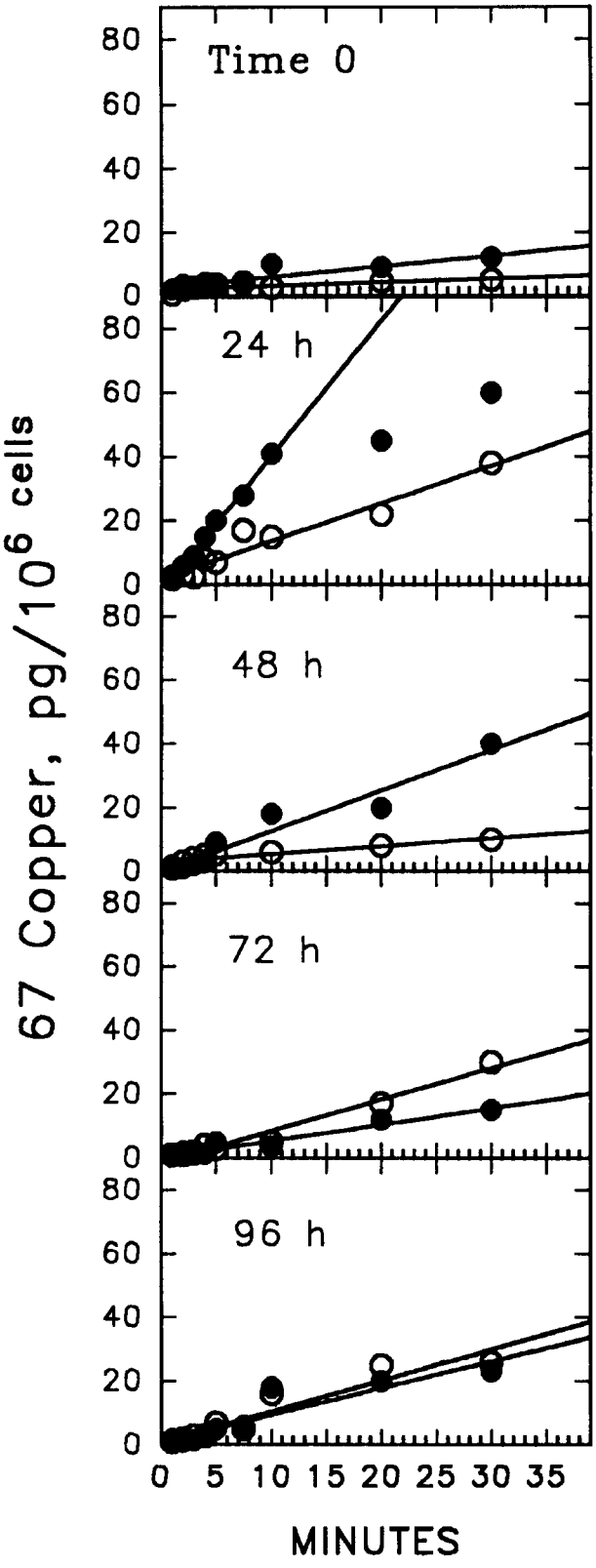

Figure 1 Copper uptake. This is one representative set of data of the uptake of ^{67}Cu from ^{67}Cu-labeled Cp from six replicate experiments. 1 × 10^{10} cells/L were incubated with 2 μmol/L ^{67}Cu-Cp for the times indicated. Uptake into cells was determined by subtracting cell-associated radioactivity at 4° C from incubations at 37° C. Rate was estimated from the slope of line created over 30 min of uptake, except at 24 hr. Then it was determined over 10 min (see text).

Table 2 Rate of ^{67}copper uptake in retinoic acid-treated and control HL-60 cells

Time	HL-60	Retinoic acid-treated
Hours	*pg ^{67}Copper/min/10^6 cells*	
0	0.33 ± 0.12[c]	0.41 ± 0.26[c]
24	0.90 ± 0.17[c]	3.41 ± 0.44[a]
48	1.15 ± 0.57[c]	2.22 ± 0.98[b]
72	1.15 ± 0.35[c]	0.59 ± 0.35[c]
96	0.95 ± 0.10[c]	0.76 ± 0.50[c]
ANOVA		
Hours	$P < 0.05$	
Treatment	$P < 0.05$	
Hours × treatment	$P < 0.05$	

Values are means and SD of six replicate experiments. Different superscripts indicate significant differences of means.
RA, retinoic acid.
Uptake was determined in the presence of 1 μmol/L retinoic acid.

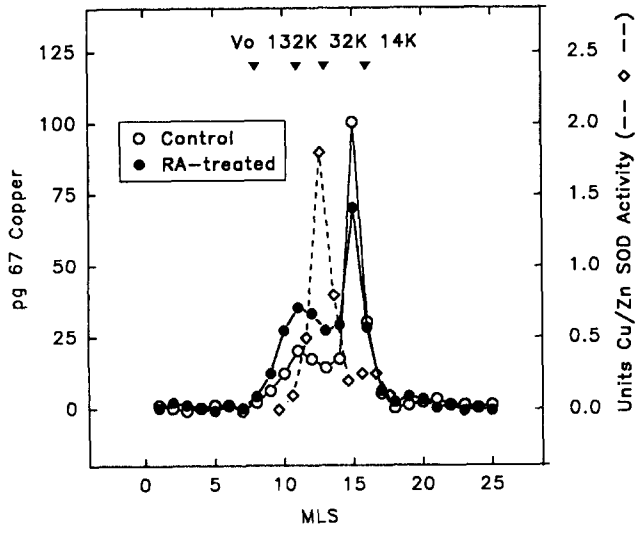

Figure 2 Gel filtration chromatography of the supernatant of ^{67}Cu-labeled RA-treated and untreated HL-60 cells. Cells were incubated for 3 hr with ^{67}Cu-Cp. RA-treated cells are depicted by closed circles and control untreated cells are the open circles. Cu/Zn SOD activity is depicted by the diamonds connected by a dashed line. This is one representative set of data from four replicate experiments.

Discussion

We showed previously that differentiated cells had more total copper than the undifferentiated cells, and that this copper was not associated with Cu/Zn-SOD or CCO.[5] This investigation of accumulation and distribution of total and radioactive copper demonstrated that uptake was regulated early during differentiation, and that the increase in total copper may be related to the appearance of a high molecular weight protein.

The level of total copper found in the supernatant of the two cell types was similar when based on cell number, yet it was twofold greater in RA-treated cells compared with untreated cells when expressed per mg cell protein. The majority of cellular copper (60 to 85%) is found in the supernatant of these cells when compared with the whole cell homogenates.

After cells were fully differentiated, the amount of ^{67}Cu radioactivity per cell equivalent (after 3 hr of exposure to ^{67}Cu-Cp) was lower in RA-treated cells compared with untreated cells. This slowing of copper accumulation after cells are differentiated may be because a new steady state of copper had been reached.

Soon after inducing the cells to differentiate (within 24 to 48 hr), RA-treated cells were able to take up copper faster than at other time points. This was a transient increase, as the uptake subsequently returned to control levels during the last 2 days of differentiation. Thus, the high level of copper found in RA-treated cells is at least partially because of an enhanced uptake early in differentiation. The magnitude of change in uptake (about twofold) is the same as the increase in total copper. RA-treated cells have twice as much copper as the untreated control cells, and uptake into RA-treated cells was double the rate of untreated cells for about 2 days.

The intracellular distribution of ^{67}Cu from Cp is unique to HL-60 cells and unlike that previously demonstrated in the K562 cell line.[11] Previous studies with K562 cells showed that about 80% of total cytosolic ^{67}Cu was incorporated into Cu/Zn-SOD, while in the HL-60 cell relatively little radioactivity was incorporated into fractions containing Cu/Zn-SOD. This could be due to a relatively smaller amount

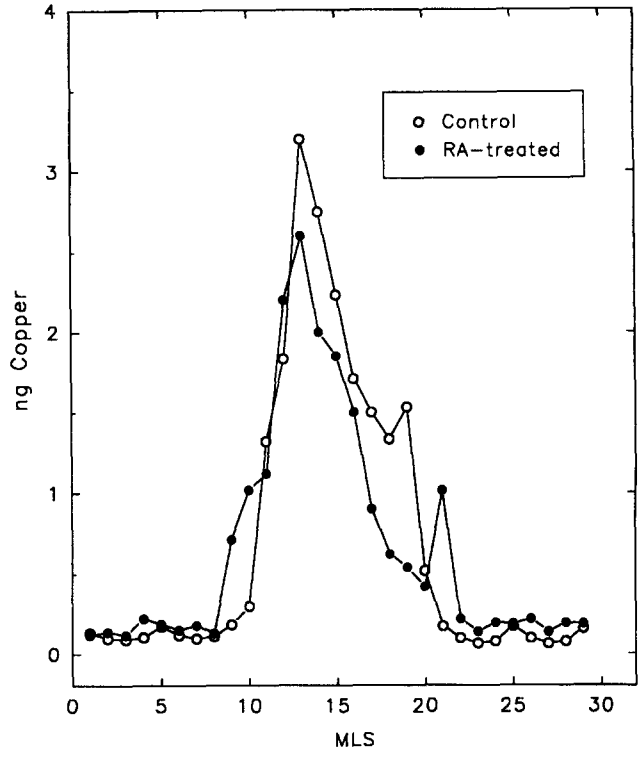

Figure 3 Copper analysis of fractions after gel filtration chromatography of the supernatant of untreated and 96 hr RA-treated cells. This is one representative set of data from four replicate experiments, and the data are expressed in cell equivalents. RA-treated cells are depicted by closed circles and control untreated cells are the open circles.

of Cu/Zn-SOD in the HL-60 cell compared with the K562 cell or slower turnover of the Cu/Zn-SOD protein in the HL-60 cells. HL-60 cells contain roughly tenfold less Cu/Zn-SOD protein[6] compared with the K562 cell.[11]

The ^{67}Cu binding proteins found in the HL-60 cell could be potentially important in the process of neutrophil development. The intracellular distribution of ^{67}Cu was found predominately in a high molecular weight protein (fraction 10) and a low molecular weight protein (fraction 15). Neither of these fractions corresponded to molecular weights of any known copper binding proteins, and therefore, their identity becomes critical to understanding copper's function in this cell type.

The radioactive peak in fraction 15 is probably not metallothionein. Fraction 15 is estimated to be about 20,000 D and metallothionein has an apparent molecular weight of 10,000 D, which would elute in fraction 20. Furthermore, HL-60 cells have low basal levels of metallothionein protein (Huber, Percival, and Cousins, 1992: unpublished data) and little mRNA for metallothionein.[12]

Neither does the copper binding protein in fraction 15 appear to be the monomer of Cu/Zn-SOD because the enzyme activity that was recovered in fraction 13 was equivalent to that found in whole cell homogenates. Further work is necessary to identify this copper binding-protein in fraction 15.

The copper binding protein in fraction 10 was estimated to have a molecular weight around 100,000 D. Fraction 10 had greater amounts of ^{67}Cu and total copper in the RA-treated cells than in the untreated cells. The protein(s) in fraction 10 may play a role in accumulating intracellular copper; however, the current results do not distinguish between a greater amount of protein or a greater amount of ^{67}Cu bound to a protein.

Ettinger recently reported a copper-binding protein with a molecular weight of 50 kD[13] that self-associates into dimers and tetramers.[14] This protein has been suggested to be involved in intracellular copper trafficking. Although the method of labeling the mouse liver cytosol preparations employed by Ettinger is in contrast to our in vivo labeling with ^{67}Cu-Cp, the high molecular weight copper-binding protein reported in these studies may be related to the 50 kD protein.

Copper is clearly important during granulopoiesis, as shown by the accumulation of copper and the regulation of uptake. The ability of an RA-treated cell to retain more copper may be due to the appearance of a copper-binding protein, specifically the high molecular weight protein. This protein may be an important protein in the differentiation process of promyelocytes towards neutrophils. We speculate that neutropenia may be related to a lack of copper for this protein.

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