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Increase of Cu,Zn-Superoxide Dismutase Activity during Differentiation of Human K562 Cells Involves Activation by Copper of a Constantly Expressed Copper-deficient Protein*

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Cu,Zn-superoxide dismutase activity, expressed on the basis of cell number, increased by 50% during sodium butyrate-induced differentiation of human K562 erythroleukemia cells. The increased enzyme activity was found to be concomitant with constant Cu,Zn-superoxide dismutase mRNA and immunoreactive protein levels and was accompanied by a rise in intracellular copper and glutathione. Incubation of K562 cell homogenates with copper caused an increase of Cu,Zn-superoxide dismutase activity which reached the levels observed after differentiation in the presence of sodium butyrate. The same treatment led to no significant activity increase in homogenates derived from differentiated cells. Externally added ceruloplasmin increased both intracellular copper levels and Cu,Zn-superoxide dismutase activity in undifferentiated cells to a level comparable with that observed after induction of differentiation. Both increments were abolished by depletion of cell glutathione. Cu,Zn-superoxide dismutase purified from control cells had both a lower k_{cat} and a lower copper content than the enzyme purified from differentiated cells. From these data we conclude that: 1) Cu,Zn-superoxide dismutase is present in K562 cells also under the form of a less active copper-deficient enzyme, 2) the extent of enzyme activation is regulated post-translationally by differential delivery of copper as a function of differentiation stage, and 3) glutathione is likely to play a role in delivering copper to the copper-deficient protein in intact K562 cells.

Aerobic life is challenged by partially reduced oxygen species (O_2^-, H_2O_2, $OH^·$) which are transiently formed during biological processes (1). Due to their higher reactivity, as compared with molecular dioxygen, they are considered to be potentially toxic to cells. Among the several enzymatic and non-enzymatic defense mechanisms that have evolved against reactive oxygen species superoxide dismutases (EC 1.15.11, SODs)[1] are thought to provide a primary line of defense by catalyzing the dismutation of the one-electron reduction product of oxygen, O_2^-, to H_2O_2 and O_2 (2). Generally, eukaryotes possess two forms of SODs: one manganese-containing enzyme that is located in the mitochondrial matrix and one copper- and zinc-containing protein which is found in the cytosol (3). In spite of their analogous enzymatic function and the fact that both proteins are encoded on nuclear genes, the two enzymes differ greatly with respect to their inducibility. Mn-SOD seems to be adaptively regulated in an oxygen-dependent fashion (4), while Cu, Zn-SOD behaves as a "housekeeping" protein in mouse and rat tissues (5) as well as in *Xenopus laevis* (6). However, the underlying mechanisms of the fine regulation of its gene are still poorly understood. Elucidation of the mechanistic patterns by which Cu,Zn-SOD activity is controlled would be of pivotal importance for a better definition of the biological role of this enzyme.

An interesting model system was recently described (7), which involves an increased Cu,Zn-SOD activity in erythroleukemia cells induced to differentiate along a pseudo-erythroid pathway. The *in vitro* erythroid cell differentiation goes along with reduction of cell growth and concomitant accumulation of hemoglobin (8, 9). It has been suggested that increased Cu,Zn-SOD activity would be likely to occur in any cellular system triggered for hemoglobin synthesis (7). In fact, enhanced hemoglobin levels might cause an increased intracellular O_2^--flux which could induce *de novo* synthesis of Cu,Zn-SOD (7).

This hypothesis has been questioned by recent findings, indicating no changes in CN^--inhibitable SOD activity, which corresponds to the Cu,Zn-enzyme, to occur during differentiation of Friend erythroleukemia cells (10). Moreover human K562 erythroleukemia cells were found to undergo a decrease in Cu,Zn-SOD activity during hemin-induced differentiation (11).

In view of these rather contradictory reports we have reinvestigated the erythroleukemia cell differentiation model with special focus on the relationship between Cu,Zn-SOD activity,

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[1] The abbreviations used are: SOD, superoxide dismutase; BSO, (*d*, *l*)-buthionine (*S*,*R*)-sulfoximine; FELC, Friend erythroleukemia cells; GSH, glutathione; HMBA, *N*,*N'*-hexamethylenebisacetamide; PBS, phosphate-buffered saline; SB, sodium butyrate; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; ELISA, enzyme-linked immunosorbent assay; FPLC, fast protein liquid chromatography.

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TABLE I
Effect of SB on Cu,Zn-SOD, Mn-SOD, catalase and lactate dehydrogenase levels in K562 cells

	Cu,Zn-SOD	Mn-SOD	Catalase	Lactate dehydrogenase
	μg/10⁶ cells	*units/10⁶ cells*	*units/10⁶ cells*	*units/10⁶ cells*
Control	0.212 ± 0.046 (n = 9)	0.123 ± 0.05 (n = 3)	9.3 ± 4.4 (n = 3)	0.58 ± 0.12 (n = 3)
SB	0.314 ± 0.074[a] (n = 9)	0.082 ± 0.004 (n = 3)	10.0 ± 3.9 (n = 3)	0.48 ± 0.08 (n = 3)

[a] Significantly different from control as evaluated by Student's t test ($p < 0.01$).

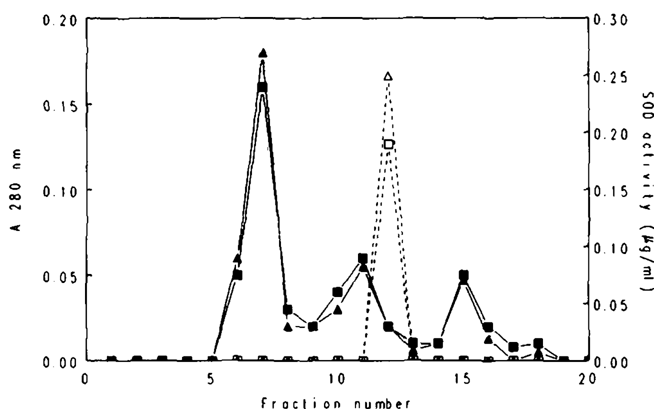

FIG. 1. **Gel filtration chromatography of 17000 × g supernatants of K562 cells.** 17,000 × g supernatants derived from 10^6 control or SB-treated K562 cells were chromatographed on a Superose 12 FPLC column operating at 0.3 ml/min. 1.2-ml fractions were collected, and Cu,Zn-SOD activity was determined polarographically in each fraction. *Solid lines:* $A_{280\,nm}$; ■, control; ▲, SB-treated cells; *dashed lines*: SOD activity; □, control; △, SB-treated cells.

protein and mRNA levels. We show that differential delivery of copper to a constantly expressed protein is a major mechanism of the regulation of Cu,Zn-SOD activity in K562 cells induced to differentiate by exposure to sodium butyrate. Different ratios between the less active copper-deficient protein and the fully active holoprotein account for the observed changes of the enzyme activity level, a case previously suggested only for yeast or animal cells subjected to copper starvation (12–15).

MATERIALS AND METHODS[2]

RESULTS

96-h exposure to SB resulted in a 50% increase of polarographically detectable SOD activity in K562 erythroleukemia cells (Table I). This activity increase was completely abolished by 3 mM CN^-, which is a specific inhibitor of Cu,Zn-SOD (2). Gel filtration chromatography of 17,000 × g supernatants derived from either control or SB-treated cells showed that SOD activity eluted as a single peak with an approximate molecular mass of 30 kDa (Fig. 1). Thus, polarographically detectable activity corresponds to the activity of the Cu,Zn-containing enzyme in both control and SB-treated cells.

Other enzymes involved in oxygen free radical detoxification such as catalase or Mn-SOD did not parallel the behavior of Cu,Zn-SOD, whereas glutathione peroxidase was below the detection limits of our analytical procedure (33) in both control and differentiated cells. Lactate dehydrogenase activity, assayed as control, was unaffected by SB exposure (Table I).

Relationship between Cu,Zn-SOD Activity and Hemoglobin Levels—In order to evaluate the possibility of a correlation between hemoglobin and Cu,Zn-SOD activity, nine subclones expressing different basal levels of hemoglobin were isolated from the original K562-A clone. Each subclone was induced to differentiate by exposure to SB. Cu,Zn-SOD activity and hemoglobin content were determined for control and differentiated cells of each subclone and plotted as shown in Fig. 2. No correlation between Cu,Zn-SOD activity and hemoglobin content could be detected using either a least squares linear regression or an iterative nonlinear regression.

As a further test, we examined FELC induced to differentiate along an analogous erythroid pathway by exposure to HMBA. This cell line failed to show a significant increase of Cu,Zn-SOD activity on a per cell basis during differentiation passing from 0.085 ± 0.008 μg/10^6 cells to 0.095 ± 0.015 μg/10^6 cells (n = 5).

The degree of hemoglobin production during the differentiation of FELC and K562 cells differed greatly as shown in Fig. 3. Thus, there was no recognizable correlation between hemoglobin levels and Cu,Zn-SOD activity when the two cell lines were compared; whereas hemoglobin increased only slightly in K562 cells (where Cu,Zn-SOD augments), FELC underwent a 23-fold increase in hemoglobin content during differentiation, but no significant alteration in Cu,Zn-SOD activity, expressed on the basis of cell number, was noticed.

Effects of SB Treatment on Metallothionein and Cytochrome Oxidase Activity in K562 Cells—In order to evaluate if increased Cu,Zn-SOD activity in K562 cells in response to SB treatment was paralleled by other copper proteins, metallothionein, which is a major copper protein in melanoma and copper-resistant hepatoma cells (25, 34), and cytochrome oxidase activity were assayed. The latter was found to increase 2.5-fold after 96-h SB exposure (3.17 ± 1.18 nmol $min^{-1}l^{-1}$/10^6 cells *versus* 7.8 ± 0.9 nmol $min^{-1}l^{-1}$/10^6 cells, n = 4).

Metallothionein, on the other hand, although present in both control and differentiated cells as demonstrated by immunoblotting (Fig. 4), was unaffected by SB treatment. FPLC fractionation of 17,000 × g supernatants and ELISA analysis of each fraction showed a broad peak of immunoreactive protein around M_r = 12000. Metallothionein was detectable also in higher molecular weight fractions due to the presence of oligomeric metallothionein species that are formed in cell extracts unless prepared under anaerobic conditions (25). The ELISA profiles indicate almost identical levels of metallothionein in both control and differentiated cells (Fig. 4).

Effect of SB on Cu,Zn-SOD mRNA and Protein Levels—Northern blot hybridization revealed the expression, in both differentiated and undifferentiated K562 cells, of the two Cu,Zn-SOD mRNA species of 0.7 and 0.9 kilobases (not shown) that have been found in other human cells (35). SB treatment was not accompanied by an altered transcription of the Cu,Zn-SOD gene as demonstrated by unaffected mRNA levels (Table II). Furthermore, Western blot analysis demonstrated that SB induced no significant changes in Cu,Zn-SOD protein levels (Table II). Moreover, about twice as much immunoreactive than enzymatically active protein was detectable in control cells, whereas no such discrepancy was observed in SB-treated cells.

Effects of Copper on SOD Activity of Cell Extracts—The present data suggest that a post-translational event could be responsible for the increase of Cu,Zn-SOD activity in differentiating K562 cells. Since the enzyme works at diffusion-

[2] Portions of this paper (including "Materials and Methods," Figs. 2, 3, and 6, and Tables III, V, and VI) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are included in the microfilm edition of the Journal that is available from Waverly Press.

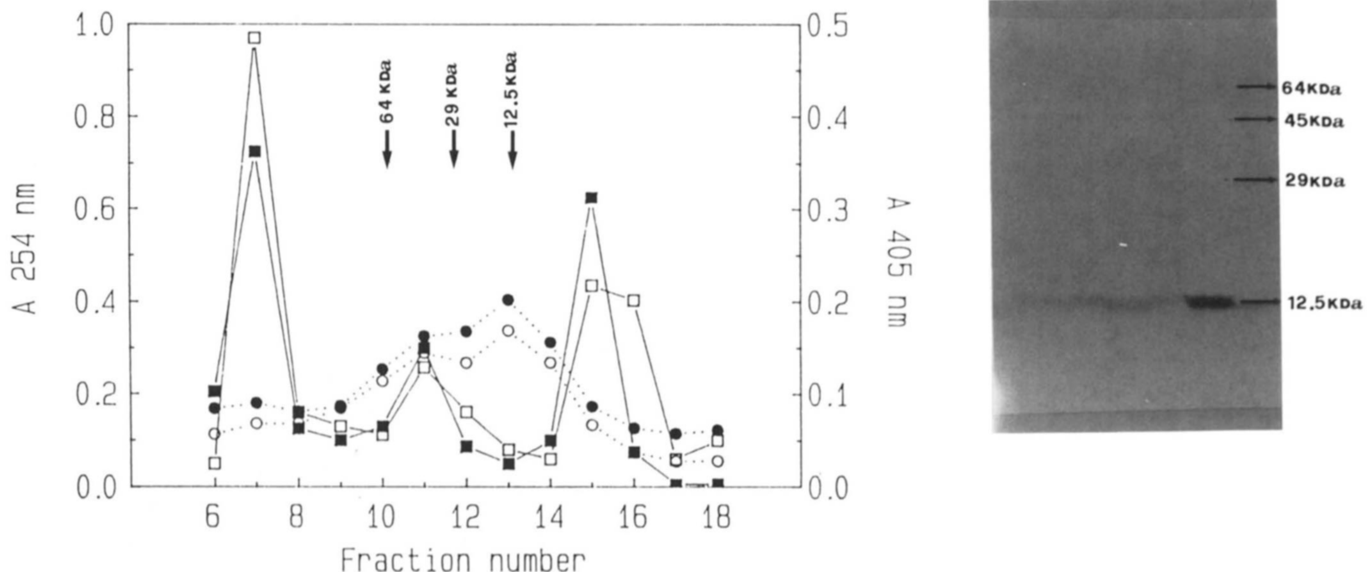

FIG. 4. **Effect of SB treatment on metallothionein content of K562 cells.** *Left panel*, FPLC analysis of metallothionein in control and differentiated K562 cells. 17,000 × *g* supernatants of 5 × 10^6 control or differentiated cells were chromatographed on a Superose 12 FPLC column as described under Fig. 1. Fractions were analyzed for metallothionein by direct ELISA using the alkaline phosphatase method ($A_{405\,min}$) as described under "Materials and Methods." Protein content of each fraction was estimated by monitoring the absorption at 254 nm. *Solid lines*: $A_{254\,nm}$; ■, control; □, SB; dashed lines: $A_{405\,nm}$; ●, control; ○, SB. The *arrows* indicate the fractions where bovine serum albumin, carbonic anhydrase, and cytochrome *c*, respectively, were eluted. *Right panel*, Western blot analysis of metallothionein in control (*lanes B* and *D*) and SB-treated K562 cells (*lanes C* and *E*), derived from two separate differentiation experiments. *Lane A*, purified rat liver MT I. The *arrows* indicate the positions of bovine serum albumin, ovalbumin, carbonic anhydrase, and cytochrome *c*, respectively, on the slab gel. Electrophoresis and Western blot were performed as described under "Materials and Methods."

TABLE II

Cu,Zn-SOD activity, mRNA, and protein levels in control and differentiated K562 cells

Column 2 refers to Cu,Zn-SOD activity after incubation of cell homogenates with 2 mM copper sulfate at room temperature followed by extensive dialysis (see "Materials and Methods").

	Cu,Zn-superoxide dismutase			
	Activity	Activity after addition of copper	mRNA	Immunoreactive protein
	μg/10⁶ cells	*μg/10⁶ cells*	*arbitrary densitometric units/10⁶ cells*	*μg/10⁶ cells*
Control	0.212 ± 0.046 (n = 9)	0.300 ± 0.053[a] (n = 6)	0.52 ± 0.06 (n = 10)	0.45 ± 0.07 (n = 3)
SB	0.314 ± 0.074[b] (n = 9)	0.307 ± 0.032[b] (n = 6)	0.49 ± 0.10 (n = 10)	0.35 ± 0.09 (n = 3)

[a] Significantly different from control as evaluated by Student's t test ($p < 0.05$).
[b] Significantly different from control as evaluated by Student's t test ($p < 0.01$).

limited rates and no activator of Cu,Zn-SOD has been described so far, it appears possible that a protein with a substoichiometric metal content be present in K562 cells and that activity increase is related to an increased delivery of copper ions to the enzyme's active site. In order to investigate this hypothesis cell homogenates were incubated with $CuSO_4$ according to a procedure that has previously been shown to replenish apoCu,Zn-SOD with copper even in the presence of competing bioligands (15). As shown in Table II Cu,Zn-SOD activity increased significantly in 17,000 × *g* supernatants of control cells after incubation with 2 mM $CuSO_4$ and subsequent extensive dialysis, reaching the levels attained after 96-h incubation of K562 cells with SB. By contrast, when 17,000 × *g* supernatants of SB-exposed K562 cells were subjected to the same experimental procedure, Cu,Zn-SOD activity was unaffected (Table II). In both cases FPLC experiments revealed that the SOD activity coeluted with Cu,Zn-SOD (not shown). Incubation with 2 mM $CuSO_4$ caused a 30% increase of Cu,Zn-SOD activity in the 17,000 × *g* supernatants of both untreated and differentiated FELC (data not shown). These data are indicative of the presence of a copper-free SOD protein in both cell lines, which was activated by copper in K562 cells during exposure to SB.

Actually, copper levels were found to increase in both whole K562 cells and in the 17,000 × *g* supernatants after exposure to SB (Table III). Moreover, by comparison with the data in Table I it can be inferred that about 70% of the copper found in the soluble protein fraction of both control and SB-treated K562 cells was bound to Cu,Zn-SOD.

Effect of Ceruloplasmin on Copper Content and Cu,Zn-SOD Activity in K562 Cells—If SB enhances Cu,Zn-SOD activity by increasing copper content of K562 cells, then loading cells with copper by alternative mechanisms should exert the same effect. In order to test this possibility we exposed K562 cells to ceruloplasmin. We chose ceruloplasmin instead of a low molecular form of copper because K562 cells are quite suscep-

TABLE IV

Effect of BSO on Cu,Zn-SOD activity, cellular copper, and protein levels of K562 cells induced to differentiate by exposure to sodium butyrate

Data are derived from one experiment representative of four, in which the same trend was observed. In column 3 the immunoreactive protein levels, determined by immunoblotting and quantified by laser densitometry, are expressed as relative values with respect to the level in 10^6 control cells.

	Cu Zn superoxide dismutase			Cellular		
	Activity	Activity after addition of copper	Immunoreactive protein	Copper	Protein	GSH
	μg/10⁶ cells	*μg/10⁶ cells*	*densitometric units*	*pmol/10⁶ cells*	*mg/10⁶ cells*	*nmol/10⁶ cells*
Control	0.186	0.375	1.0	14	0.250	4.6
SB	0.322	0.372	1.0	26	0.296	9.1
SB + BSO	0.253	0.292	0.8	18	0.242	0.1

tible to toxicity of copper salts (36). Furthermore, this cell line was recently shown to have ceruloplasmin receptors and to efficiently accumulate copper intracellularly from externally added ceruloplasmin (37).

Fig. 5 shows that ceruloplasmin added to the growth medium increased both cellular copper and Cu,Zn-SOD activity. The latter reached a plateau value after 22-h incubation, which was comparable to the value obtained after 96-h exposure to SB.

Glutathione Content and Cu,Zn-SOD Activity in K562 Cells—Recently glutathione was shown to be a most efficient copper donor to copper-free SOD *in vitro* (38). Glutathione content of K562 cells increased during SB treatment (Table IV). Depletion of glutathione through inhibition of its synthesis by exposure to BSO (29) inhibited the SB-induced increase of Cu,Zn-SOD activity (Table IV). Both SB-triggered differentiation and metabolic depletion of glutathione over a 96-h period have pleiotropic effects on cell metabolism which could eventually interfere with each other, thereby complicating the interpretation of experimental data. In fact, BSO abolished the SB-induced increase of cellular protein (Table IV). This process turned out to affect Cu,Zn-SOD protein levels and, consequently, reconstitutability of the protein by copper (Table IV). Therefore, the observed decrease of Cu,Zn-SOD activity in SB + BSO-treated cells, with respect to cells exposed to SB alone, was, at least in part, due to effects of BSO on protein synthesis and not exclusively to GSH depletion. To circumvent these difficulties we checked the effects of 22-h exposure of undifferentiated K562 cells to BSO alone on the ceruloplasmin-induced increase of Cu,Zn-SOD activity. In this case BSO completely abolished both the increase of enzyme activity and of cellular copper without affecting Cu,Zn-SOD protein levels (Fig. 5). In fact, the protein in BSO-treated cells proved to be fully reconstitutable by copper (Fig. 5).

Characterization of Cu,Zn-SOD Purified from Control and SB-exposed K562 Cells—Cu,Zn-SOD was purified 400-fold from K562 cells using ion exchange and gel filtration chromatography (Table V). Since the metal content does not affect the isoelectric point of the protein (39) this purification procedure is expected not to discriminate between copper-containing and copper deprived forms. The proteins derived from control or differentiated cells produced the same pattern of four bands on both silver- and activity-stained nondenaturing PAGE (Fig. 6, *lanes 1, 2, 4, and 5*). An identical pattern was displayed by pure recombinant human Cu,Zn-SOD (Fig. 6, *lane 3*). On SDS-PAGE all samples gave one single band with an apparent relative molecular mass of 20 kDa (Fig. 6, *lanes 7–9*).

Table VI shows that the protein purified from SB-treated cells had a higher k_{cat} and a higher copper content than that

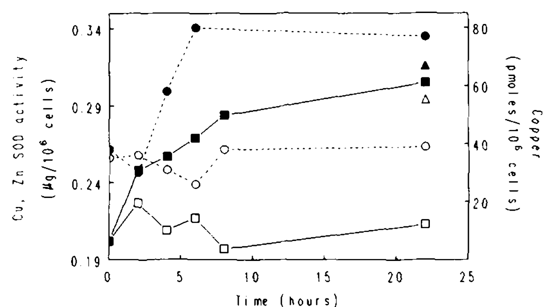

FIG. 5. **Effect of ceruloplasmin on copper content and SOD activity of K562 cells.** K562 cells, either control or cells in which GSH was depleted through inhibition of its synthesis by BSO, were incubated at a cell density of 10^6/ml in the presence of 1 μM ceruloplasmin as described under "Materials and Methods." Glutathione content was 5.0 nmol/10^6 cells in control cells and <0.1 nmol/10^6 cells in BSO-treated cells. At the indicated time points, cells were harvested and assayed for SOD activity and copper content. *Solid lines*: Cu,Zn-SOD activity; control (■), BSO-treated cells (□). Cu,Zn-SOD activity after incubation with 2 mM copper and dialysis; control (▲), BSO-treated cells, (△). *Dashed lines*: copper content of 17,000 × *g* supernatants; control (●), BSO-treated cells (○). No changes in cellular copper content or Cu,Zn-SOD activity were detectable in control or BSO-treated cells in the absence of ceruloplasmin. Data are from one representative experiment. The same trend was observed in two more experiments.

purified from control cells. Both increments were in the same order of magnitude as the activity increase observed during SB treatment of K652 cells, indicating that the copper-lacking form of control cells was quantitatively copurified with the holoprotein by our isolation procedure. The catalytic constant of the protein purified from control cells increased, reaching $1.15 \times 10^9\ M^{-1}s^{-1}$, when incubated for 1 h in the presence of stoichiometric amounts of Cu-GSH (38). The catalytic constants of the two protein samples were identical to each other when expressed on the basis of copper content. This suggests that in both cases the metal was quantitatively bound to the active site coordination sphere. Furthermore, when zinc was determined in the protein sample purified from control K562 cells a zinc:protein stoichiometry of 3.9:1 was found. In view of the copper content of 0.92 mol/mol of protein of the same sample, this figure is suggestive of zinc being bound to the copper site in the copper-deficient Cu,Zn-SOD.

DISCUSSION

Increase of Cu,Zn-SOD activity in K562 cells during *in vitro* induced differentiation was not paralleled by Mn-SOD or catalase (EC 1.11.1.6) activities (Table I). Thus, although SB caused an increase of intracellular protein in K562 cells (see "Materials and Methods" in the Miniprint), augmented Cu,Zn-SOD levels are not simply a reflection of an indiscrim-

inate effect of SB on enzymes involved in oxygen free radical metabolism.

Enhancement of Cu,Zn-SOD activity was specific for differentiated K562 cells and not a general feature of *in vitro* erythroid cell differentiation. In fact, FELC failed to show any appreciable increase of Cu,Zn-SOD activity, expressed on the basis of cell number, after differentiation with HMBA. Moreover Cu,Zn-SOD activity thus expressed proved to be unrelated to intracellular hemoglobin levels (Figs. 2 and 3).

Still, an unchanged Cu,Zn-SOD content may have different physiological effects in control and differentiated FELC due to the decrease of cellular protein during HMBA treatment (see "Materials and Methods"). As a matter of fact, differentiated FELC have a higher Cu,Zn-SOD activity/total protein ratio than control cells which may be regarded as an effective contribution to the cell's capability to face enhanced hemoglobin levels as previously suggested (7).

Cu,Zn-SOD activity paralleled the intracellular copper concentration in K562 cells and was found to account for about 70% of cytosolic copper in both control and SB-treated cells. On the other hand no changes in metallothionein content were observable (Fig. 4). Metallothionein levels are known to directly reflect the intracellular copper concentration (40), which is thought to regulate this protein at the transcriptional level (41). Presumably the changes in copper content during SB-induced differentiation of K562 cells were too small to cause *de novo* synthesis of metallothionein which was found to be mainly zinc-loaded in this cell line.[3] It is conceivable that the observed more subtle changes of the cellular copper merely exert post-translational effects, thus yielding "mature" copper proteins. As an example, cytochrome *c* oxidase subunit assembly is known to be positively affected by copper (42), which could explain the fact that we found cytochrome *c* oxidase activity to increase 2.5-fold in K562 cells during SB exposure.

In the case of Cu,Zn-SOD evidence for post-translational activation of a copper-free protein by copper during SB-induced differentiation of K562 cells was obtained by the following experimental results: (*a*) increased activity; (*b*) constant mRNA levels; (*c*) constant immunoreactive protein levels, higher than the activity levels in control cells; (*d*) augmented activity in homogenates incubated with copper, but no observable effects of copper on activity of homogenates from SB-treated cells; (*e*) increased cellular copper levels after differentiation with SB; and (*f*) isolation from control K562 cells of a Cu,Zn-SOD with a lower k_{cat} and a lower copper:protein ratio than the protein isolated from SB-treated cells.

The isolation procedure (Table V) has been performed using a limited amount of cells, due to the intrinsic difficulties of producing large quantities of differentiated cells, which stop growing after 24 h in the presence of SB. Therefore, no spectroscopic characterization has been done. However, both the reported catalytic constants and copper:protein ratios provide direct evidence for the presence of a Cu,Zn-SOD with substoichiometric copper content in K562 cells. The substantially identical electrophoretic pattern of the samples purified from either cell type in both protein and activity-stained PAGE (Fig. 6) is in line with the magnitude of the differences of copper content (50%) and the likely presence of zinc bound to the copper site of Cu,Zn-SOD in control K562 cells.

This latter finding recalls recent reports regarding *Escherichia coli* Mn-SOD (43, 44). This protein was isolated also in a catalytically inactive, iron-containing form, suggesting a role of this metal in post-translational regulation of Mn-SOD activity. As the reconstitution procedures of apoMn-SOD or Fe-Mn-SOD by added Mn differ, the latter requiring a mild denaturation step, these authors could infer the occupancy of the Mn-SOD active site by a metal other than manganese, from the reconstitution behavior of the protein in cell-free extracts (43). This may not be the case for Cu,Zn-SOD as displacement of other physiological metals, in particular zinc, from the copper-binding site by copper ions is a kinetically feasible process *in vitro* (45). In fact, any putative occupant of the copper site did not produce differences between the *in vivo* and the *in vitro* reconstitution behavior of Cu,Zn-SOD from K562 cells, as increase of cell copper by SB-induced differentiation or externally added ceruloplasmin and copper addition to cell extracts produced exactly the same activity increase (Table II and Fig. 5).

Some details of the overall picture of the regulation of Cu,Zn-SOD in K562 cells show interesting analogies to previous reports. In fact, a lack of correlation between enzyme activity and mRNA levels was observed in hepatoma cell lines (46), HL-60 cells during myeloid differentiation (47, 48), and yeast grown under different metabolic conditions (15). Moreover, a post-transcriptional regulation has been proposed for Cu,Zn-SOD in rat lung exposed to hyperthermia (48).

The existence of a copper-deficient Cu,Zn-SOD has been repeatedly reported for red blood cells (12, 13) or aortas (14) of animals held on a copper-deficient diet. In the case reported here, however, a copper-deprived SOD was detected under conditions that may be regarded as closer to the physiological ones.

We were able to reconstitute the copper-deficient Cu,Zn-SOD in intact undifferentiated K562 cells by extracellularly added ceruloplasmin (Fig. 5). This process turned out to be related to the cellular glutathione status; GSH-depletion not only impeded copper saturation of Cu,Zn-SOD in the presence of extracellular ceruloplasmin (Fig. 5) but it also inhibited the ceruloplasmin-mediated increase of total intracellular copper. These findings suggest a GSH-sensitive regulatory event in copper homeostasis of K562 cells, in particular as far as maturation of Cu,Zn-SOD is concerned. In fact, GSH has been shown to be a very efficient copper donor to apoSOD (38). Furthermore, copper overload in copper-resistant hepatomas was shown to be accompanied by transient residence of the metal in the glutathione pool (49).

The enhanced uptake of copper in differentiated cells probably is a reflection of the profound changes in growth rate and cellular metabolism produced by SB (9). It was beyond the scope of this work to investigate how SB increases copper uptake of K562 cells. However, it is very likely that this process was mediated by ceruloplasmin since we were able to detect micromolar amounts of copper and immunoreactive ceruloplasmin levels in fetal calf serum, which was used as a constituent of the growth medium (not shown). Actually, differentiation of K562 cells has been shown to go along with an increased expression of ceruloplasmin receptors (50, 51).

It is appealing to speculate that the copper-deficient Cu,Zn-SOD found in K562 cells may play a role in copper homeostasis, possibly by finely tuning the intracellular copper status at low total concentrations and on a short timescale acting as a buffer for "free" copper which may be harmful to the cell. This role would be complementary to that of metallothionein, which was found to convey resistance to uncontrolled reactivity of copper at higher concentrations and on a long time scale (52). Glutathione-copper complexes may turn out to play a role as copper donors to either system. Work in progress in this laboratory suggests that this is in fact the case for

[3] C. Steinkühler, O. Sapora, M. T. Carrì, W. Nagel, L. Marcocci, M. R. Ciriolo, U. Weser, and G. Rotilio, unpublished observations.

metallothionein as well, in line with the chemical evidence for this role already reported in the case of Cu,Zn-SOD (38).

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Supplementary Material to:
Increase of Cu, Zn Superoxide Dismutase Activity during Differentiation of Human K562 Cells involves Activation by Copper of a Constantly Expressed Copper-Deficient Protein.

by

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MATERIALS AND METHODS

Chemicals. RPMI 1640 minimal growth medium, fetal calf serum, glutamine, penicillin and streptomycine were purchased from Flow. N,N' hexamethylenebisacetamide, butyric acid and xanthine were obtained from Sigma. NADH, NADPH, xanthine oxidase, cytochrome c, glutathione and glutathione reductase were products of Boehringer. All other chemicals were obtained from Merck.

All chemicals were of analytical grade purity. Buffer solutions were prepared with double distilled water equilibrated with a Chelex -100 resin (Biorad) to remove trace amounts of copper. Copper content of buffer solutions was kept below 0.2 µM by this procedure.

Cell Culture. K562 erythroleukemia cells (clone A) were purchased from the American Type Culture Collection and grown in RPMI 1640 supplemented with 10% fetal calf serum, 2 mM glutamine and 100 U/ml penicillin and streptomycin in humidified atmosphere containing 5% CO_2 at 37°C.

Friend erythroleukemia cells (clone 3CL8vitro), kindly supplied by Dr. F. Belardelli, Istituto Superiore di Sanità, Rome, Italy, were grown under the same conditions with the exception that the growth medium contained 5% fetal calf serum.

Differentiation of K562 cells was induced by addition of 2 mM sodium butyrate as previously described [9]. Friend erythroleukemia cells were induced to differentiate by addition of 5 mM HMBA according to established protocols [7].

After 96±4 h (K562 cells) or 120±4 h (FELC) cells were harvested by centrifugation, extensively washed with PBS (20 mM sodium phosphate, 140 mM NaCl pH 7.4) and resuspended in the same buffer. Vitality was estimated by Trypan blue exclusion and cell density was determined using a Neubauer chamber or a Coulter counter. Cell suspensions were sonicated for 30 s at 20 W using a model B12 Branson sonifier and centrifuged at 17000 x g for 15 minutes. The 17000 x g supernatants were used for enzyme determinations.

Hemoglobin and Enzyme Assays. Spectrophotometric determinations were performed on a Perkin-Elmer Lambda 9 spectrophotometer equipped with a Lambda computing unit.

Hemoglobin was estimated by monitoring the Soret absorption band at 415 nm in the 17000 x g supernatants assuming an absorption coefficient of 125 $mM^{-1}cm^{-1}$ per heme [16]. MnSOD was measured with the xanthine oxidase/cytochrome c assay in the presence of 3 mM CN^- as described elsewhere [17]. Determination of catalase was performed by a UV-method according to Lück [18]. Cytochrome C oxidase was assayed as previously described [19]. Cu, Zn SOD activity was determined polarographically at pH 9.6 [20] using a model 466 Amel polarographic unit. Data were expressed in µg/ml with reference to purified bovine Cu, Zn SOD. Control experiments demonstrated no interference of SB on the accuracy of the assay. MnSOD falls to neglectable levels (below 5% of total activity) under the assay conditions; moreover the method is insensitive to the presence of hemoglobin [21].

In some experiments Cu, Zn SOD was measured after prior incubation of 17000 x g supernatants of control or differentiated cells with 2 mM $CuSO_4$ for 3h at 25 C and subsequent 1: 2000 dialysis for 16 h against 50 mM phosphate buffer pH 7.8 containing 0.1 mM EDTA. Typically, copper concentration in the samples after dialysis was 10^{-4} M. This copper concentration was shown not to affect the accuracy of the assay. Furthermore, unspecific SOD-mimetic activity of low Mr Cu-chelates was avoided due to the scavenging of Cu by EDTA in the assay system [20].

Protein was determined by the method of Lowry et al. [22]. Protein content of K562 control cells was 0.250±0.017 mg/10^6 cells and 0.353±0.037 mg/10^6 cells (n=3) in sodium butyrate-treated cells, whereas HMBA-treatment of FELC resulted in a decrease of cellular protein content (0.118±0.014 mg/10^6 cells in control vs. 0.0812±0.008mg/10^6 cells (n=3) in differentiated FELC). For this reason all activities were calculated on the basis of cell number (U or µg per 10^6 cells).

Metal Determinations. For copper determination, 10^8 cells were harvested by centrifugation, and washed three times with PBS. The cell pellet was resuspended in 400 µl PBS. After determination of cell density, 300 µl of the resulting cell suspension were transfered to acid-washed vials. Upon addition of 700 µl 65 % HNO_3 the vials were sealed and kept at room temperature for 7 days in order to allow complete hydrolysis to occur. Control samples consisted of PBS treated in the same way. For determination of copper in the 17000 x g supernatants, cells were disrupted by sonication and centrifuged as described above.

For determination of zinc the sample was hydrolized for 16 hours at room temperature in 6 N HNO_3. Copper and zinc determinations were performed with a Perkin-Elmer 3030 atomic absorption spectrometer equipped with a graphite furnace.

Electrophoresis and Immunoblotting. Antisera against bovine Cu, Zn SOD raised in rabbits were a gift from Dr. Franco Marmocchi, University of Camerino, Italy. They proved to efficiently react with human Cu, Zn SOD on nitrocellulose filters, giving a linear response between 50ng and 400ng of purified human recombinant Cu, Zn SOD protein which was a gift of Biotechnology General, Rehovot, Israel.

17000 x g supernatants derived from an equal number of cells were electrophoresed on two polyacrylamide gels according to Laemmli [23]. One gel was stained with Coomassie blue, the other one was subjected to a standard Western blot. Cu, Zn SOD was detected on the nitrocellulose filters after incubation with the specific antisera by the horseradish-peroxidase method (Biorad). Quantitation was performed by laser densitometry of the bands using an LKB Ultrascan XL unit.

Total RNA Extraction and Northern Blot Analysis. Total RNA was extracted from 10^7 K562 cells by the guanidine isothiocyanate method as described elsewhere [24]. The Northern blot was performed according to standard protocols [6], using the whole coding sequence for human Cu, Zn SOD (a kind gift of Dr. Y. Groner, Weizmann Institute of Science, Rehovot, Israel) as a probe upon [^{32}P]dATP labeling by random primer extension. Quantitation of the autoradiograms was obtained by densitometric scanning with a LKB Ultroscan XL laser densitometer coupled with an LKB 2400 GelScan software package. Exposure times of Kodak films varied in order to obtain quantitable hybridisation signals. In every experiment the two major ribosomal RNAs served as internal standard to evaluate total RNA yield per cell and to normalize the Cu, Zn SOD hybridisation signal. Data are given in arbitrary densitometric units, expressing normalized areas of integrated peaks from densitometric scanning.

Detection of Metallothionein by Immunoblotting. SDS-polyacrylamide gel electrophoresis and immunoblot of 0.5x10^6 K562 cells was performed as previously described [25]. The blotting efficiency was examined by staining the nitrocellulose filter with Ponceau S protein dye after protein transfer. The nitrocellulose filters were incubated with murine monoclonal antibody (mAb 1-8h) against monomeric rat liver MT-I which has been demonstrated to react with metallothionein of human melanoma cells [26]. Metallothionein was visualized with an alkaline phosphatase-conjugated antibody as previously described [25].

FPLC. 17000 x g supernatants of 5x10^6 or 10^6 K562 cells were chromatographed on a Superose 12 column equilibrated with PBS, which contained 0.1 % 2-mercaptoethanol when metallothionein was assayed. Fractions of 1.2 ml were collected. Protein content was estimated by monitoring the absorbance at 254 nm or at 280 nm. Cu, Zn SOD activity was measured polarographically in each fraction. Alternatively, microtiter plates were coated with the fractions eluted from Superose 12 and analyzed for metallothionein by ELISA [27]. Each determination was performed in quadruplicate (S.E.M. was less than 5 %).

Incubation of K562 cells with ceruloplasmin and depletion of GSH. Sheep ceruloplasmin, purified as previously described [28], was a kind gift from Dr. L. Calabrese (University La Sapienza, Rome, Italy). The protein had a copper stoichiometry of 5:1 and an A610/A280 ratio of 0.045, which is indicative of a high degree of purity. K562 cells were grown to a cell density of 0.5 x 10^6/ml, splitted and to one half 2 mM BSO was added to deplete cellular glutathione [29]. After 22 h cells were harvested by centrifugation and resuspended in fresh complete medium or complete medium containing 10 µM BSO (GSH-depleted cells) at a cell density of 10^6 /ml. To control and GSH-depleted cells 1 µM ceruloplasmin was added. At different times cells were collected by centrifugation and extensively washed with PBS. Cu, Zn SOD activity and copper were determined as described above. GSH was determined before and at the end of each experiment to control depletion efficiency, using the method of Griffith [30]. Under our experimental conditions GSH fell to undetectable levels in BSO-exposed cells and no de novo synthesis could be detected during the incubation with ceruloplasmin.

Purification of Cu Zn SOD from K562 cells. K562 cells were grown and harvested as described above. After cell disrupture by sonication the resulting homogenates were centrifuged at 17000 x g and stored under liquid nitrogen. 17000 x g supernatants derived from 10^9 K562 cells, either control or sodium butyrate-treated, were pooled and centrifuged for 1 h at 105000 x g. The resulting supernatants were dialysed overnight against 5 mM sodium phosphate pH 7.4 and applied to a 1.5 x 20 cm DE-52 (Whatman) column equilibrated with the same buffer. The column was washed with 50 ml 5 mM sodium phosphate pH 7.4 and Cu, Zn SOD was eluted with a linear NaCl gradient ranging from 0 to 80 mM. The activity containing fractions were pooled, concentrated to 200 µl by ultrafiltration using Amicon YM membranes with a nominal cutoff of 10 kDa and chromatographed on a 1.5 x 32 cm Superose 12 FPLC column equilibrated with PBS and operating at 0.3 ml/min. The Cu, Zn SOD containing fractions were pooled and equilibrated with 5 mM sodium phosphate pH 6.3 by several concentration-dilution cycles using an Amicon stirred ultrafiltration cell. The sample was then concentrated to 150 µl and chromatographed on a 0.5 x 7 cm CM-52 column (Whatman) equilibrated with the same buffer. After this step the Cu, Zn SOD proteins derived from either control or SB-treated cells were approximately 95 % pure. To remove a minor contaminant the samples were electrophoresed on a 7.5 % polyacrylamide gel run under non-denaturing conditions at 4°C. The Cu, Zn SOD containing bands were excised from the gel, transferred into 0.5 x 10 cm glass tubes into which a 0.5 cm 12 % polyacrylamide gel had been polymerized. Cu, Zn SOD was electroeluted overnight into a dialysis tube using a tube gel apparatus run at 20 mA. Copper and zinc content of the purified protein was determined by atomic absorption spectroscopy. Protein determination of the purified protein samples was done by computerized laser scanning densitometry of Coomassie or silver stained gels with reference to pure human recombinant Cu, Zn SOD, as previously described [31]. The catalytic constants were determined polarographically and calculated using a simplified Koutecky equation [20]. Activity staining of gels was performed as described elsewhere [32].

TABLES

TABLE III. EFFECT OF SB ON THE COPPER CONTENT OF WHOLE K562 CELLS AND 17000xg SUPERNATANTS. Data are mean values ± standard deviation of 6 different experiments

	COPPER (pmoles/10^6 cells)	
	WHOLE CELLS	17000xg SUPERNATANTS
Control	38.4±14.0	18.5±3.5
SB	66.8±23.4	27.5±4.0

TABLE V. PURIFICATION OF Cu, Zn SOD FROM K562 CELLS. Data refer to the purification from control K562 cells. The same protocol was employed for the purification from differentiated K562 cells, which yielded 15 µg of pure Cu, Zn SOD.

FRACTION	VOLUME (ml)	PROTEIN (mg/ml)	ACTIVITY (µg/ml)	SPECIFIC ACTIVITY (µg/mg)	TOTAL ACTIVITY (µg)
17000 x g supernatant	6	24	21.6	1.1	130
105000 x g supernatant	5.5	-	23	-	126
DE-52	0.185	-	540	-	100
Superose 12	0.160	-	437	-	70
CM-52	0.042	-	1309	-	55
PAGE	0.410	0.131	53.7	410	22

TABLE VI CATALYTIC CONSTANTS AND COPPER CONTENT OF Cu Zn SOD PURIFIED FROM CONTROL OR SB-TREATED K562 CELLS Under the experimental conditions a $k_{cat} = 2.0 \times 10^9 M^{-1}s^{-1}$ was determined for pure bovine Cu, Zn SOD. Data are mean values of at least three determinations. Reproducibility was better than ± 5%.

	kcat ($M^{-1} s^{-1}$)	moles Cu/ mole protein	kcat/Cu ($M^{-1} s^{-1}$)
Control	0.82×10^9	0.92	0.89×10^9
SB	1.30×10^9	1.44	0.90×10^9

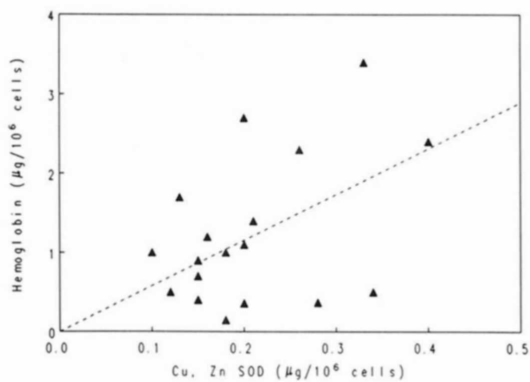

Figure 2. Relationship between Cu, Zn SOD activity and hemoglobin content of different K562 cell clones induced to differentiate in the presence of SB.
9 different clones were obtained by serial dilution from the original K562 cell line, induced to differentiate and processed. Hemoglobin and Cu, Zn SOD activity were determined as described under Materials and Methods. Cu, Zn SOD activity and hemoglobin were not correlated, as calculated from linear regression data (r=0.4448).

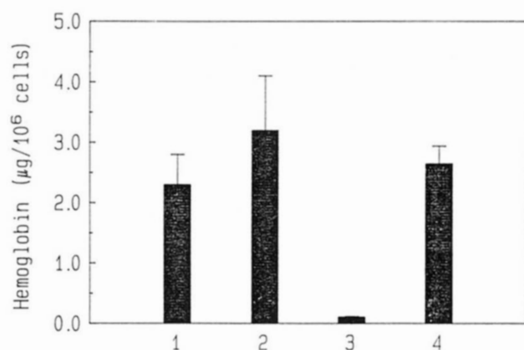

Figure 3. Effect of differentiation on hemoglobin content of K562 cells and FELC.
K562 cells and FELC were induced to differentiate by exposure to SB or to HMBA, respectively. Hemoglobin content was estimated by monitoring the absorbance of the Soret band at 415 nm in the 17000xg supernatants. 1. K562 control; 2. K562, SB-treated; 3. FELC control; 4. FELC, HMBA-treated. Data are means ± standard deviation of 4 different experiments.

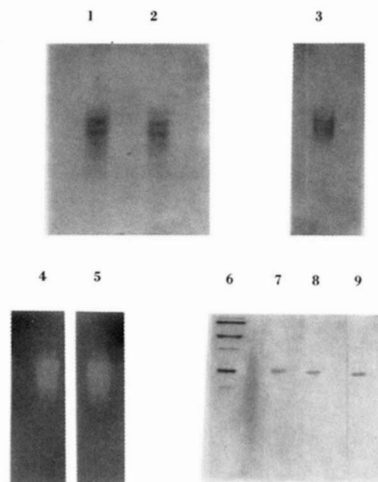

Figure 6. Polyacrylamide gel electrophoresis of Cu, Zn SOD purified from K562 cells.
Lanes 1-5 electrophoresis performed on 7.5 % gels run under nondenaturing conditions at 20 mA/gel. Lanes 1-3 were stained with silver stain, lanes 4 and 5 were activity stained. Lane 1: 1 µg Cu, Zn SOD from control K562 cells; lane 2: 1 µg Cu, Zn SOD from SB-treated K562 cells; lane 3: 1 µg purified human recombinant Cu, Zn SOD; lane 4: 0.8 µg Cu, Zn SOD from control K562 cells, lane 5: 0.8 µg Cu, Zn SOD from SB-treated K562 cells. Lanes 6-8: electrophoresis performed on a 15 % gel in the presence of SDS at 20 mA/gel, silver staining. Lane 6: molecular weight markers (biorad): phosphorylase b (97 kDa), bovine serum albumin (64 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), trypsin inhibitor (21 kDa), lysozyme (14 kDa); lane 7: 0.5 µg Cu, Zn SOD purified from control K562 cells; lane 8: 0.5 µg Cu, Zn SOD purified from SB-treated K562 cells; lane 9: 0.5 µg pure human recombinant Cu, Zn SOD.