Chinese hamster fibroblasts overexpressing CuZn-superoxide dismutase undergo a global reduction in antioxidants and an increasing sensitivity of DNA to oxidative damage

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Transfection of a CuZn-superoxide dismutase (SOD) expression vector into V79 Chinese hamster cells produced clones in which CuZn-SOD activities were 2.2–3.5-fold higher than in the parental cells. An overall moderate reduction of glutathione peroxidase, glutathione reductase and glucose-6-phosphate dehydrogenase activities and of both GSSG and total glutathione

levels was found. In one particular clone the catalase activity was also reduced. The pro-oxidant status established by the lower level of antioxidant defence rendered the SOD-overexpressing cells more sensitive to the production of 8-oxo-2'-deoxyguanosine by hydrogen peroxide. The data are discussed in terms of a model resembling the bacterial sox RS system.

INTRODUCTION

The importance of superoxide dismutase (SOD) in aerobic organisms was foreseen many years ago [1] and seems to have culminated with the discovery that the basis for the familial form of amyotrophic lateral sclerosis relies on certain types of mutations in the CuZn-SOD gene [2,3].

Efforts to understand the mechanism of toxicity of the superoxide anion (O_2^-) have led to several proposals and have been updated recently [4]. With the progress of recombinant DNA technology and the possibility of gene transfection to cells or to organisms, the role of SOD has become more amenable to experimentation. Cells lacking SOD genes [5] or SOD expression [6] have been produced, and many lines overexpressing SOD have been engineered by gene transfection. In general, the results of experiments in this line have revealed an apparent fine balance of antioxidant enzymes in the cell; changes in the expression of SOD seem to interfere with the expression of other proteins, even those not related to the cellular homeostasis of reactive oxygen species. In bacteria [7] and in mammalian cells [8,9], overexpression of SOD in cells bearing extra copies of the SOD gene produced a higher sensitivity to reactive oxygen species. In these cases, it has been postulated that SOD overexpression would lead to an accumulation of H_2O_2 , one of the products of dismutation of O_2^- [7–9]. However, an excess of SOD should decrease the steady-state O_2^- level but not alter the steady-state H_2O_2 concentration [10]. It is possible, however, that changes in other enzymes activities produce a pro-oxidant status in the cell. Indeed, it has been observed that overexpression of SOD in *Escherichia coli* causes a decrease in glucose-6-phosphate dehydrogenase (G6PD) and glutathione reductase activities, as well as a decrease in NADPH and GSH levels [10]; this seems to be a consequence of a reduction in the O_2^- level, sensed by the sox RS regulon [11].

In mammalian cells, overexpression of CuZn-SOD caused different responses in different systems. A compensatory increase in glutathione peroxidase (GPX) occurred as a consequence of the introduction of the CuZn-SOD expression vector into L-cells, neuroblastoma cells and primary mouse cells [12,13]. In JB6 cells, overexpression of CuZn-SOD resulted in increased DNA

breakage upon exposure to the xanthine/xanthine oxidase system [8]. Finally, transfection of the CuZn-SOD expression vector into 3T3 murine fibroblasts resulted in two classes of transfectants, characterized by the presence or absence of an increase in endogenous GPX [14].

We set out to investigate the consequences of the transfection of a CuZn-SOD expression vector into V79 Chinese hamster fibroblasts, in two respects: the sensitivity of DNA to damage produced by pro-oxidant status and changes in other antioxidant defences in the cell. Interestingly, a trend for a moderate but significant decrease in the levels of several antioxidants was observed, similar to what had been noticed in bacteria [10] and distinct from what has been observed in mammalian cells [12–14]. As an apparent consequence of that, the DNA became more susceptible to accumulate lesions on exposure of the cells to H_2O_2 .

MATERIALS AND METHODS

Cell culture

M8 is a clone derived from V79 Chinese hamster lung fibroblasts. The cells were grown in Dulbecco's modified Eagle's medium (DMEM), pH 7.0, supplemented with 10% (v/v) fetal calf serum, 472 units of penicillin/ml and 94 μ g of streptomycin/ml. The cells were cultured in humidified CO_2 /air (1:19) at 37°C.

Cell transfection

M8 cells were transfected by the calcium phosphate method [15] and selected for geneticin resistance. The CuZn-SOD expression vector p-GSOD-SVneo (SV is simian virus) [9] was provided by Dr. Y. Groner (Weizmann Institute of Science, Israel). Four overexpressing clones were isolated and named pGSOD1–pGSOD4. These cells were cultured in the presence of 500 μ g/ml of geneticin except for a 48 h period before the assay.

Cell extracts

Mn-SOD assay was performed in cell lysates obtained by treating trypsinized and PBS-washed cells with PBS containing 0.2%

(v/v) Nonidet P40 for 5 min on ice, followed by centrifugation at 11 800 *g* for 15 min at 4 °C.

For GSSG determination, cells were treated with trypsin and resuspended in ice-cold DMEM, without serum. After a 5 min centrifugation at 500 *g* at 4 °C, the pellet was resuspended in 300 μ l of ice-cold 1 M HClO₄/2 mM EDTA solution and the insoluble material was sedimented by centrifugation at 8000 *g* for 10 min at 4 °C. This procedure was done as fast as possible to avoid GSH oxidation.

Additional enzyme assays were performed in cell extracts obtained as follows. Cells were treated with trypsin, resuspended in DMEM containing 10 % (v/v) fetal calf serum and washed three times with PBS. The cells were sonicated in an ice-water bath for three 15 s bursts (on a Microson ultrasonic cell disruptor; Heat Systems, Farmingdale, NY, U.S.A.) with 30 s cooling intervals and the resulting suspension was centrifuged for 10 min at 11 800 *g*. The supernatant was used for protein determination [16], total glutathione and enzyme assays.

Enzyme assays

SOD activity was determined from the inhibition of hydroxylamine oxidation by O₂⁻ [17], with Mn-SOD being differentiated from CuZn-SOD by the addition of 5 mM cyanide. Catalase activity was assayed by measuring the rate of decrease in H₂O₂ absorbance at 240 nm [18]. Cytosolic GPX and glutathione reductase activities were assayed essentially as described by Sies et al. [19]. G6PD was assayed as described by Glock and McLean [20].

Total glutathione determination

To the cell extract an equal volume of 2 M HClO₄/4 mM EDTA was added and the precipitated proteins were sedimented by centrifugation at 8000 *g* for 10 min at 4 °C. The supernatant was neutralized with 2 M KOH/0.3 M Mops and the insoluble KClO₄ was removed by centrifugation under the same conditions. For the spectrophotometric determination, 910 μ l of the cell extract supernatant or of the standard glutathione solution, in phosphate/EDTA buffer, was mixed with 50 μ l of 4 mg/ml NADPH in 0.5 % (w/v) NaHCO₃, 20 μ l of 6 units/ml glutathione reductase in phosphate/EDTA buffer and 20 μ l of 1.5 mg/ml 5,5'-dithiobis-(2-nitrobenzoic acid) in 0.5 % NaHCO₃. The increase in absorbance measured at 412 nm under these conditions is directly related to the sum of reduced and oxidized forms of glutathione in the cell extract [21]. Total glutathione content was expressed in terms of mg per g of protein.

GSSG determination

The acidic extract was neutralized to a final pH of 6.0 by the addition of 2 M KOH/0.3 M Mops and the insoluble KClO₄ was removed by centrifugation at 8000 *g* for 10 min at 4 °C. The remaining supernatant was split into two samples for the determination of both total glutathione and GSSG. For GSSG determination 4-vinylpyridine was added to a final concentration of 0.1 % (v/v) and then incubated for 1 h at room temperature [22]. At this concentration 4-vinylpyridine can react with all GSH without interfering with the GSSG determination (results not shown). Both total glutathione and GSSG content were determined as described for total glutathione determination. GSSG content was expressed in terms of GSSG/total glutathione as a weight ratio.

H₂O₂ consumption

Eight hours after plating, the cells (4 × 10⁵/dish) were washed twice with PBS/Ca (PBS containing 0.8 mM CaCl₂) and 10 ml of 0.2 mM H₂O₂ in PBS/Ca were added. After the indicated times at 37 °C, aliquots (100 μ l) were removed to determine the H₂O₂ concentration [23].

Northern-blot analysis

Northern-blot analysis was performed essentially as described previously [24]. RNA extraction was carried out as described by Birnboim [25].

Determination of the 8-oxo-2'-deoxyguanosine content of DNA in H₂O₂-treated cells

Cells (6.3 × 10⁶/plate) were seeded in 24.5 × 24.5 cm plates. Forty-eight hours later the cells were semiconfluent and the medium was removed. After two washes with PBS/Ca the cells were exposed to 100 ml of 50 mM H₂O₂ in PBS/Ca for 30 min at 37 °C. Upon removal of this solution the cells were lysed with an extraction solution containing 0.2 % SDS, 5 mM trans-1, 2-diaminocyclohexane-N,N,N',N'-tetraacetic acid (CDTA), 1 mM LiCl, 50 mM Tris/HCl (pH 8.0) and 1 M urea. To the resulting lysate 100 μ g/ml of proteinase K was added and incubation proceeded for 1 h at 45 °C. The suspension was vigorously passed twice through a gauge 22 needle in order to produce DNA fragmentation. The DNA was precipitated by the addition of 0.55 vol. of isopropanol and centrifugated at 10 000 *g* for 10 min at 0 °C. After washing with 70 % ethanol, the pellet was resuspended in 5 ml of a solution containing 1 mM CDTA, 10 mM Tris/HCl, pH 7.5. After complete solubilization, DNase-free RNase was added to the final concentration of 50 μ g/ml and the solution was incubated at 37 °C for 10 min. This was followed by two extractions: the first with an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1, by vol.) and the second with an equal volume of chloroform/isoamyl alcohol (24:1, v/v). The resulting aqueous phase was then mixed with 0.1 vol. of 3 M sodium acetate, pH 4.8, and 3 vol. of 95 % ethanol and the precipitated DNA was centrifugated at 10 000 *g* for 10 min at 0 °C. After washing with 70 % ethanol the pellet was dried and finally dissolved in the digestion buffer (20 mM sodium acetate, pH 4.8).

The 8-oxo-2'-deoxyguanosine content of DNA was determined by electrochemical detection of the modified nucleoside after HPLC separation of DNA enzyme hydrolysates, as described by Shigenaga et al. [26]. The separation was performed using a Shimadzu LC10AD solvent-delivery unit (Shimadzu, Tokyo, Japan) equipped with a Rheodyne (Cotati, CA, U.S.A.) injector and a Spherex 5C18 (250 × 4.6 mm) column (Phenomenex, Torrance, CA, U.S.A.). The mobile phase consisted of filtered and degassed 50 mM KH₂PO₄ buffer (pH 5.5)/methanol (9:1, v/v) at a flow rate of 1 ml/min. Samples were analysed by UV detection using a Waters 484 unit (Waters, Milford, MA, U.S.A.) followed by electrochemical detection using a Shimadzu L-ECD-6A unit (Shimadzu, Tokyo, Japan) at an oxidation potential of +0.6 V.

For quantitative analysis of 8-oxo-2'-deoxyguanosine and 2'-deoxyguanosine, sample peaks were compared with standard curves obtained on the same day by the injection of known amounts of standards.

RESULTS

CuZn-SOD-overexpressing clones were isolated by transfecting the parental cell line (M8) with an expression vector (p-GSOD-

Table 1 CuZn-SOD activity in transfected cells

The values correspond to the mean $\pm$ S.D. for the number of determinations in parentheses.

Cell lines	CuZn-SOD activity (units/mg of protein)
M8	5.6 $\pm$ 0.3 (5)
pGSOD1	12.9 $\pm$ 0.6 (5)
pGSOD2	12.6 $\pm$ 1.4 (5)
pGSOD3	12.6 $\pm$ 0.4 (5)
pGSOD4	19.9 $\pm$ 3.8 (5)

Table 3 8-oxo-2'-Deoxyguanosine content of DNA in H₂O₂-treated cells

8-oxo-2'-Deoxyguanosine content of DNA is expressed in pmol 8-oxo-2'-deoxyguanosine/mg of DNA. Cells were exposed to PBS/Ca containing 50 mM H₂O₂ for 30 min at 37 °C. The values correspond to the mean $\pm$ S.D. for the number of determinations in parentheses (**P* < 0.05 compared with H₂O₂-treated M8 cells as determined by Student's *t* test).

Cell lines	Control	H ₂ O ₂
M8	6.5 $\pm$ 1.7 (5)	51.1 $\pm$ 7.4 (4)
pGSOD1	6.5 $\pm$ 1.1 (4)	61.0 $\pm$ 3.0 (4)
pGSOD2	8.3 $\pm$ 1.6 (4)	67.8 $\pm$ 2.1 (4)*
pGSOD3	6.1 $\pm$ 0.1 (4)	65.8 $\pm$ 0.3 (4)*
pGSOD4	8.9 $\pm$ 0.4 (3)	89.7 $\pm$ 0.1 (4)*

Figure 1 Northern blot analysis

The RNAs were probed with a ³²P-labelled human CuZn-SOD cDNA (top) or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene (bottom).

SVneo [9]) containing the entire human SOD-1 gene in addition to a neo transcriptional unit. Four geneticin-resistant transfectant clones were isolated and expanded. They all resemble the parental cell line (M8) in growth rate and morphological characteristics (results not shown). The four clones showed an increase in CuZn-SOD activity (2.2–3.5 times the parental cell line activity;

Table 1). Stationary CuZn-SOD mRNA concentration in transfected cells was determined by Northern-blot analysis (Figure 1) using a ³²P-labelled human CuZn-SOD cDNA as a probe. It is evident that the amount of stationary mRNA is significantly higher in the four pGSOD clones when compared with M8 parental cells, demonstrating that the increase in CuZn-SOD activity is due to an increase in mRNA levels.

It is important to note that statistically significant decreases (*P* < 0.05) occurred for some antioxidants in different clones (Table 2). It is also interesting that there is an inverse relationship between the CuZn-SOD levels and the other enzyme activities. The clone pGSOD4, which displays the highest SOD activity, also has the highest decrease in GSSG, total glutathione, glutathione reductase and G6PD and is the only clone that presents a decrease in catalase activity. The only exception is GPX whose activity is only slightly (not statistically significantly) decreased in pGSOD4. No changes in Mn-SOD activities occurred in these clones.

In order to investigate the role of O₂⁻ in H₂O₂-induced DNA damage we have determined the 8-oxo-2'-deoxyguanosine content of DNA in these cells when submitted to H₂O₂ stress. CuZn-SOD-overexpressing clones presented higher contents (statistically different in clones pGSOD2, pGSOD3 and pGSOD4) of H₂O₂-induced 8-oxo-2'-deoxyguanosine content when compared with the parental cell line (Table 3). Although 50 mM H₂O₂ might seem to be a very harsh condition, it must be considered that the high number of cells per plate (~5 × 10⁷) causes rapid decomposition of H₂O₂ (50% in ~6 min) so that the average concentration of H₂O₂ to which the cells are exposed is much lower than 50 mM [27]. Indeed, the cells remained normal in

Table 2 Relative antioxidant contents

Enzyme activities, total glutathione contents and GSSG/total glutathione ratios are expressed relative to M8 cells. The values correspond to the mean $\pm$ S.D. for the number of determinations indicated in parentheses. M8 cells antioxidant contents: Mn-SOD, 0.565 $\pm$ 0.095 units/mg of protein; catalase, 27 $\pm$ 1 units/mg of protein; GPX, 2.42 $\pm$ 0.09 units/g of protein; glutathione reductase, 99 $\pm$ 2 units/g of protein; G6PD, 145 $\pm$ 1 units/g of protein; total glutathione, 6.7 $\pm$ 1.1 mg/g of protein and GSSG/total glutathione, 0.0114 $\pm$ 0.0002 g of GSSG/g of total glutathione (**P* < 0.05 compared with M8 cells as determined by Student's *t* test).

Cell lines	Mn-SOD	Catalase	GPX	Glutathione reductase	G6PD	Total glutathione	GSSG/total glutathione
M8	1.00 $\pm$ 0.17 (11)	1.00 $\pm$ 0.04 (8)	1.00 $\pm$ 0.04 (6)	1.00 $\pm$ 0.02 (8)	1.00 $\pm$ 0.01 (7)	1.00 $\pm$ 0.17 (15)	1.00 $\pm$ 0.02 (14)
pGSOD1	0.90 $\pm$ 0.03 (11)	1.00 $\pm$ 0.04 (8)	0.86 $\pm$ 0.02 (6)	0.90 $\pm$ 0.01 (6)*	0.87 $\pm$ 0.01 (6)*	0.74 $\pm$ 0.07 (11)*	0.80 $\pm$ 0.05 (9)*
pGSOD2	1.08 $\pm$ 0.12 (11)	1.04 $\pm$ 0.04 (8)	0.77 $\pm$ 0.07 (6)*	0.97 $\pm$ 0.04 (6)	0.89 $\pm$ 0.01 (6)*	0.59 $\pm$ 0.17 (11)*	0.77 $\pm$ 0.03 (9)*
pGSOD3	0.99 $\pm$ 0.10 (11)	1.11 $\pm$ 0.08 (8)	0.85 $\pm$ 0.05 (6)*	0.92 $\pm$ 0.01 (6)*	0.84 $\pm$ 0.02 (6)*	0.78 $\pm$ 0.22 (14)	0.76 $\pm$ 0.07 (9)*
pGSOD4	1.08 $\pm$ 0.05 (11)	0.52 $\pm$ 0.04 (8)*	0.94 $\pm$ 0.05 (6)	0.77 $\pm$ 0.01 (6)*	0.79 $\pm$ 0.01 (6)*	0.32 $\pm$ 0.12 (10)*	0.73 $\pm$ 0.13 (10)*

Figure 2 Consumption of H_2O_2 by the transfected cells

Symbols: ○, M8; ●, pGSOD1; □, pGSOD2; ■, pGSOD3; △, pGSOD4.

appearance during the treatment. No statistically significant differences in 8-oxo-2'-deoxyguanosine were seen in the clones at H_2O_2 concentrations lower than 25 mM (results not shown). No differences among these clones were seen in H_2O_2 -induced DNA strand breaks as measured by alkaline sucrose gradient analysis (results not shown).

We also determined the H_2O_2 consumption of these cell lines (Figure 2). Both pGSOD2 and pGSOD3 present the same rate of H_2O_2 consumption as the parental cell line. Therefore, the differences in DNA 8-oxo-2'-deoxyguanosine contents of these cell lines (Table 3) are not merely caused by differences in H_2O_2 consumption rate. This observation could probably reflect differences in intracellular antioxidant compartmentalization, especially in the nucleus. As expected, the pGSOD4 clone exhibits the lowest rate of H_2O_2 consumption due to its reduced content of catalase and total glutathione.

DISCUSSION

Many mammalian systems overexpressing CuZn-SOD have been described [8,9,14,28–35]. In some of these cases the increase in CuZn-SOD was reported to be protective against oxidative stress [9,33,36]. In other cases overexpression of SOD produced a higher sensitivity to reactive oxygen species [8,9]; this phenomenon was also observed in bacteria [7,37]. It has been postulated that SOD overexpression would lead to accumulation of H_2O_2 , one of the products of dismutation of O_2^- [7–9]. However, an excess of SOD should decrease the O_2^- steady-state level but not alter the steady-state H_2O_2 concentration [10]. Since the clones we obtained showed reduced GSSG contents (Table 2), it is unlikely that they present higher H_2O_2 steady-state levels in non-stressing conditions. It seems also possible that the overexpression of CuZn-SOD may cause alterations in other systems by so far unknown mechanisms.

In the present work we attempted to clarify some aspects of the mechanism(s) involved in the changes in the antioxidant repertoire caused by CuZn-SOD overexpression. Chinese hamster lung fibroblasts (V79-M8 clone) overexpressing CuZn-SOD were derived by transfection with a CuZn-SOD expression vector. All clones obtained presented higher CuZn-SOD activities and mRNA levels (Table 1 and Figure 1).

These clones showed a global decrease in other antioxidant species (Table 2) and, with the exception of GPX, this decrease seemed to be related to the CuZn-SOD activity, suggesting

control of a group of genes by O_2^- . It is known that in lower organisms both O_2^- and H_2O_2 can induce many genes [38–40]. It has recently been demonstrated that reactive oxygen species can also be involved in gene expression systems in mammalian cells. For instance, H_2O_2 and O_2^- can activate the transcription factor NF- κB [35] and induce the expression of cell growth factors [41–43]. H_2O_2 also induces activation of the iron responsive element-binding protein responsible for post-transcriptional control of iron homeostasis [44,45] and is necessary in platelet-derived growth factor signal transduction [46].

It is interesting that the overall changes in antioxidant enzyme activities resemble that found in the sox RS system in bacteria, in that overexpression of SOD leads to a decrease in the activity of G6PD and pro-oxidant status is established [10,11]. To our knowledge this has not been detected previously in experiments in which other mammalian cells were transfected with SOD expression vectors. In most of those cases an increase in GPX was detected [14]. The reasons for these discrepancies are not obvious. One possibility is to consider that changes in O_2^- steady-state levels may be dealt with by two gene-control systems in mammalian cells. One of them would be similar to the sox RS system. Under conditions of overexpression of SOD and low O_2^- levels, down-regulation of this system would lead to decreases in G6PD, GSH and other antioxidant enzymes, as observed in the present study. A pro-oxidant status would then be observed, and this might be responsible for the discrete increase in sensitivity of DNA to H_2O_2 in terms of production of 8-oxo-2'-deoxyguanosine (Table 3). The lack of GSH could lead to an increase in H_2O_2 which, depending on the level and on the particular cell line, could activate another system leading to increases in GPX, MnSOD, catalase and other enzymes. This second level of pro-oxidant status would be more severe and could explain situations in which spontaneous lipid peroxidation is observed [9].

It is therefore possible that an increase in H_2O_2 concentration occurs in cells overexpressing SOD, not as a consequence of the dismutation itself, but resulting from a discrete pro-oxidant status which is established by a gene control system that is responsive to O_2^- .

It is also interesting to notice that these results seem to obviate the need of O_2^- as an iron reducing species in the production of 8-oxo-2'-deoxyguanosine, a DNA lesion produced by the OH radical. Indeed, had this been the case we should notice a decrease in these damages by H_2O_2 in cells overexpressing SOD. Thus, although it seems clear that the Fenton reaction is responsible for DNA damage produced under oxidative stress [47,48], the need for a Haber–Weiss reaction in which Fe(II) is recycled by O_2^- seems to be dismissed. This possibility has been anticipated by others, considering the high GSH concentration normally found in the cell [4].

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