V79 Chinese-hamster cells rendered resistant to high cadmium concentration also become resistant to oxidative stress

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Chinese hamster cells (V79) resistant to high concentrations of Cd^{2+} in the medium were obtained by using the procedure of Beach & Palmiter [(1981) *Proc. Natl. Acad. Sci. U.S.A.* **78**, 2110–2114], which in mouse led to amplification of metallothionein (MT) genes and to an enrichment in cellular MT. The Cd-resistant V79 clones isolated were significantly more resistant than parental cells to oxidative stress by extracellular H_2O_2 or a mixture of H_2O_2 and superoxide anion (O_2^-) generated by xanthine oxidase plus acetaldehyde. On a per-cell basis, there was no difference between the two cells in their total H_2O_2 -decomposing or O_2^- -dismutating activity. The most likely explanation is that an enrichment in MT content in the Cd-resistant cells was responsible for this effect, because of the antioxidant properties already described for this protein.

INTRODUCTION

Metallothioneins (MTs) are proteins of low molecular mass, in general consisting of about 60 amino acids, one third of which are cysteine residues (Hamer, 1986). Their synthesis is homeostatically regulated in cells and organisms exposed to heavy metals. They strongly bind heavy metals through clusters of thiolate bonds (Hamer, 1986). Their function is still a matter of debate (Karin, 1985), but a role in metal metabolism or detoxification is strongly suggested by the ability of MT to both bind to, and be induced by, heavy-metal ions.

More recently the possibility has been raised that MT plays an antioxidant role, owing to its high content of cysteine (Thornalley & Vasak, 1985). In fact MT has a strong scavenger activity against hydroxyl radicals ($\text{OH}^\bullet$), the apparent bimolecular rate constant being of the order of $10^{12} \text{ M}^{-1} \cdot \text{s}^{-1}$ (Thornalley & Vasak, 1985).

We set about testing whether MT may afford protection to cells against oxidative stress. Recently we have shown that human and rodent fibroblasts exposed to H_2O_2 or to a mixture of H_2O_2 plus superoxide anion (O_2^-) undergo DNA damage, lethal events and sister chromatid exchanges mediated by the Fenton reaction (Hoffmann *et al.*, 1984; Mello-Filho & Meneghini, 1984, 1985; Mello-Filho *et al.*, 1984; Larramendy *et al.*, 1987), $\text{OH}^\bullet$ radical being the ultimate agent to hit the target(s). A cell enriched with MT could be less vulnerable to the effects of oxidative stress if in fact this protein acts *in vivo* as an efficient $\text{OH}^\bullet$ scavenger. The approach we used to enrich the cells with MT was that described by Beach & Palmiter (1981), in which, by a stepwise increase in Cd concentration in the medium, they obtained a progressive selection of mouse cells resistant to the metal and enriched in MT and its gene. We found that in fact Cd-resistant cells exhibited a significant increase in resistance to oxidative stress.

MATERIALS AND METHODS

Cells

To obtain a Cd-resistant clone we started from clone M8 of V79 Chinese-hamster lung fibroblasts, originally provided by Dr. M. Taylor from the University of Indiana. The cells were routinely grown in Dulbecco's modified Eagles medium, pH 7.0, supplemented with 10% (v/v) fetal-calf serum, 472 units of penicillin/ml and 94 μg of streptomycin/ml. The cells were kept in humidified CO_2 /air (1:19) at 37 °C.

To determine cell killing, 200 cells were plated on 3.5 cm-diameter Petri dishes and 10 h later submitted to treatment in 2 ml of PBS with (xanthine oxidase treatment) or without (H_2O_2 treatment) 2% fetal-calf serum, for 30 min at 37 °C in the dark. After that the cells were grown for 6 days in Dulbecco's modified medium containing 10% fetal-calf serum, fixed, and staining with Crystal Violet. Survival was scored as the ability of a cell to form a colony of at least 20 cells.

Selection of Cd-resistant cells

V79M8 cells were grown in medium containing increasing concentrations of CdSO_4 and ZnCl_2 (always at a 2:1 ratio) beginning with 10 μM - Cd^{2+} . The increase in Cd/Zn concentrations was in steps of 10 μM for Cd and 5 μM for Zn, and was carried out when the cells were bearing well the previous concentrations of Cd/Zn; that is, capable of reaching confluence. After 6 months, when cells were growing in 220 μM -Cd and 110 μM -Zn, clones were isolated. These were routinely grown in medium containing 220 μM - Cd^{2+} and 110 μM - Zn^{2+} . This procedure was adapted from that described by Beach & Palmiter (1981).

H_2O_2 decomposition by the cells

Cells (500 000) were plated on 40 mm-diameter Petri

Abbreviations used: MT, metallothionein; PBS, phosphate-buffered saline (8.1 mM- Na_2HPO_4 /1.47 mM- KH_2PO_4 /1.68 mM-KCl/137 mM-NaCl, pH 7.0); SOD, superoxide dismutase; DTPA, diethylenetriaminepenta-acetic acid; O_2^- , superoxide anion; $\text{OH}^\bullet$, hydroxyl radical; drf, dose reduction factor.

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dishes. After 6 h they were washed with PBS and received 5 ml of 10^{-4} M- H_2O_2 in PBS containing 1 mM- Ca^{2+} . The cells remained in the dark at 37 °C for different times, after which 100 μl aliquots were removed to determine H_2O_2 concentration by the method of Cotton & Dunford (1973).

Superoxide dismutase (SOD) activity

The method of Marklund (1976) was used, in which the decomposition of O_2^- was monitored spectrophotometrically at 240 nm; $(5-10) \times 10^6$ cells were trypsin-treated, centrifuged at 800 *g* and resuspended into 2.0 ml of PBS plus 10^{-5} M-DTPA. After three cycles of freezing-thawing in acetone/solid CO_2 and a water bath at 37 °C respectively, the lysate was centrifuged at 800 *g* and the supernatant was employed for determination of SOD activity, using potassium superoxide as substrate and human Cu,Zn-SOD as standard.

Under the conditions of this assay, 1 unit is defined as the amount of enzyme which affords a pseudo-first-order rate constant of O_2^- disappearance equal to 0.1 s^{-1} . A unit of human Cu,Zn-SOD in this assay corresponds to 0.030 unit in the conventional assay in which xanthine oxidase and cytochrome *c* are employed (Marklund, 1976).

Protein in the homogenates was determined by the method of Hartree (1972).

Materials

DTPA, xanthine oxidase and Cu,Zn-SOD were from Sigma; $3\text{CdSO}_4 \cdot 8\text{H}_2\text{O}$ and ZnCl_2 were analytical-grade reagents from Merck.

Metaphase chromosomes

Cells were incubated for 3 h with 0.1 μg of colchicine/ml, trypsin-treated and resuspended in 0.075 M-KCl, where they remained for 12 min at 37 °C. After addition of an equal volume of a cold methanol/acetic acid (3:1, v/v) the cells were centrifuged at 800 *g* and resuspended in the same cold methanol/acetic acid solution. Metaphase chromosomes were spread over microscope slides, air-dried and stained with 3.5% Giemsa for 5 min.

Protein electrophoresis

Discontinuous gradient gels were used as described by Lin & McCormick (1986). Separation gels consisted of a linear polyacrylamide gradient [7.5–30% (w/v) total acrylamide]; the proteins were run in the native state and stained at the end of the run with silver.

To obtain cell extracts, 2.5×10^6 cells were trypsin-treated, washed by centrifugation in PBS and resuspended in 100 μl of 0.025 M-Tris/0.192 M-glycine buffer, pH 8.3. Three steps of freezing-thawing (acetone/dry ice–37 °C water bath) were then performed to achieve cell lysis.

RESULTS

V79M8 Chinese-hamster cells were grown over several months in medium containing stepwise increasing concentrations of CdSO_4 . We ended up with cells which could grow in the presence of 220 μM - CdSO_4 , albeit slowly. At this stage clones were isolated and tested for resistance to Cd as shown in Fig. 1. In this experiment, ZnCl_2 is present in the medium at a concentration

Fig. 1. Resistance to Cd of V79M8 (○) V79Cd1 (●) and V79Cd2 (■) cells

The cells were plated in the presence of the indicated concentrations of CdSO_4 and half these concentrations of ZnCl_2 . Bars indicate the s.d. for three determinations. Plating efficiencies in this experiment for the three cell strains were 68, 71 and 76% respectively.

Fig. 2. Electrophoresis of proteins from Cd1 (lane 2) and M8 (lane 1) cells

The gel was stained with silver. A portion (100 μg) of protein from each cell extract was loaded on the gel. The identification of the bands as MTs is based on their typical migration characteristics under these experimental conditions (Lin & McCormick, 1986).

corresponding to half the CdSO_4 concentration. Without this compensation with ZnCl_2 the survival curves were all shifted leftwards (results not shown). Two of these clones, Cd1 and Cd2, exhibited a much higher resistance to Cd than did the parental clone M8. The karyotypes of clones

Fig. 3. Survival of V79M8 (○) and V79Cd1 (●) cells exposed to H_2O_2

The bars indicate the S.D. for three determinations. Plating efficiencies under these conditions were 66 and 64 % for V79M8 and V79Cd1 respectively.

Fig. 4. Survival of V79M8 (○), V79Cd1 (●) and V79Cd2 (■) cells in xanthine oxidase plus acetaldehyde (3.75 mM)

Survivals for these three cell strains after exposure to acetaldehyde alone were 87.5, 100 and 100 % respectively. Survival after exposure to xanthine oxidase alone was 100 % in all cases.

Fig. 5. Decomposition of extracellular H_2O_2 by V79M8 (○) and V79Cd1 cells (●)

Cd1 and M8 were investigated, and in both cases most of the metaphases exhibited 21 chromosomes, the difference being a slight increase in heterogeneity: in M8 cells 75 % of the metaphases had 21 chromosomes, whereas in Cd1 cells this value was 48 %. This is a completely different situation from that obtained in mouse cells, where resistance to Cd was accompanied by an increase in the number of chromosomes to tetraploidy (Beach & Palmiter, 1981). We did not determine whether an amplification for MT genes like that which occurs in the case of mouse cells had taken place. However, the experiment of Fig. 2 clearly shows the greater difference in the pattern of electrophoresed proteins between M8 and Cd1 cells. In fact, two bands on the range expected for MT under the conditions employed (Lin & McCormick, 1986) are clearly visible after staining only in the case of Cd1 cells. The presence of more than one band is not surprising, since mammals exhibit multiple forms of MT. These results show that the Cd-resistant cells have a much higher content of MT than the parental cells, probably attributable to gene amplification, as is the case with mouse cells (Beach & Palmiter, 1981).

When Cd1 and M8 cells were exposed to H_2O_2 , the former exhibited a significant increase in resistance when compared with the parental cells (Fig. 3). The ratio between the slopes of the linear portion of the curves is 2.5 and represents the dose reduction factor (drf). This means that the H_2O_2 concentration necessary to produce the same number of lethal events is 2.5 times higher for Cd1 than for M8 cells. In four experiments essentially the same results were obtained, the mean of the drf being 2.78 ± 0.27 . The survival of two Cd-resistant clones in the xanthine oxidase/acetaldehyde system was also assayed (Fig. 4). This is a system which produces a mixture of O_2^- plus H_2O_2 , and we have previously shown that it has the same effect as H_2O_2 in cell killing, since external O_2^- plays no role in terms of producing lethal events (Mello-Filho & Meneghini, 1985). Therefore it is not surprising that both Cd-resistant clones, Cd1 and Cd2,

Fig. 6. Activity of SOD in V79M8 (○) and V79Cd1 (●) cells

The inset depicts the protein content as a function of the number of cells. Points are from two separate experiments.

were much more resistant to the xanthine oxidase/acetaldehyde system than were the parental M8 cells.

The resistance to H_2O_2 exhibited by Cd-resistant cells could be due to increases in the activity of cellular antioxidant enzymes, like catalase, glutathione peroxidase and SOD. In the experiment of Fig. 5 the decomposition of extracellular H_2O_2 promoted by M8 and Cd1 cells was shown to be exactly the same on a per-cell basis, implying that the Cd1 cells have not changed their intracellular activities of catalase and glutathione peroxidase compared with the parental M8 cells. The total activity of intracellular SOD was measured by the method of Marklund (1976), and the results (Fig. 6) show a 32% decrease in SOD specific activity in Cd1 cells as compared with the parental M8 cells (0.201 ± 0.017 unit/ μg of protein and 0.297 ± 0.008 unit/ μg of protein respectively). However, the protein content increased 41% from M8 to Cd1 cells as shown in the inset to Fig. 6 (77.7 ± 2.3 $\mu\text{g}/10^6$ cells and 109 ± 1.8 μg proteins/ 10^6 cells respectively), and therefore the total SOD activities on a per-cell basis are indistinguishable in M8 and Cd1 cells (23.1 ± 1.3 units/ 10^6 cells and 22.0 ± 2.2 units/ 10^6 cells respectively).

DISCUSSION

We have shown that V79 cells selected for Cd resistance are significantly more resistant than the parental cells to oxidative stress. To obtain these cells we have basically followed the protocol described by Beach & Palmiter (1981) and we have shown, as did those authors, that the V79Cd resistant cells are enriched with MT. The assumption that an enrichment in MT is responsible for the resistance to oxidative stress in Cd-resistant cells is reasonable, because this protein has strong $\text{OH}^\bullet$ -scavenging properties *in vitro* (Thornalley & Vasak, 1985) and may also donate an H atom to a radical target, restoring it to an undamaged state (Greenstock *et al.*, 1987). In experiments using *o*-phenanthroline as an

iron chelate, we have determined that the loss of colony-forming inflicted by H_2O_2 or the xanthine oxidase system is produced by an iron-mediated Fenton reaction (Mello-Filho & Meneghini, 1985). Therefore the possibility must also be considered that the protection afforded by MT is due to Fe chelation by this protein into a form that is not active as Fenton reactant. The formation of an Fe-MT complex *in vitro* has already been described (Good & Vasak, 1986). Alternatively one may have a MT-mediated replacement of Fe by Cd at sites close to the target for lethal events, thus preventing a site-specific Fenton reaction from occurring; such Fe $\leftrightarrow$ Cd exchange induced by MT has been described as occurring in cell membranes (Thomas *et al.*, 1986). As to which is the mechanism involved in the antioxidant properties of MT *in vivo*, $\text{OH}^\bullet$ scavenging, reduction of the radical target or iron removal, this can only be answered after further investigation.

Heavy metals, including Cd, are known to induce other proteins besides MT in mammalian cells. These include other heat-shock proteins (Levinson *et al.*, 1980; Catalbiano *et al.*, 1986; Shelton *et al.*, 1986) and a 30–35 kDa protein not induced by heat shock (Keyse & Tyrrell, 1987). This 30–35 kDa protein is also induced by H_2O_2 (Keyse & Tyrrell, 1987). At present one cannot rule out the possibility that these proteins participate in the resistance to oxidative stress developed in Cd-resistant cells. However, it is noteworthy that a single pretreatment of the parental M8 cells with Cd under conditions which should lead to induction of these proteins did not render these cells more resistant to oxidative stress (results not shown). In order to develop such resistance the slow stepwise increase of Cd concentration in the medium, which in mouse cells led to MT-gene amplification, had to be adopted. Certainly further investigation is required to pinpoint the protein(s) involved in the increased resistance to oxidative stress (other than catalase, SOD and glutathione peroxidase) and to determine the mechanism involved in such resistance.

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