Metallothionein protects DNA from oxidative damage

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Metallothionein (MT) is a potent hydroxyl radical scavenger but its antioxidant properties *in vivo* have not been defined. Most of the recent results indicate that it does not afford protection to cells against the lethal action of oxidative stress. However, the possibility that MT confers protection against oxidative damage to a specific cellular target, such as DNA, had not been considered. We compared V79 Chinese hamster cells enriched in and depleted of MT in terms of DNA-strand scission. Zinc induces an increase in MT content of V79 Chinese hamster cells, without concomitant increase in the GSH level. These induced cells are more resistant to the production of DNA-strand scission by H_2O_2 than the parental cells. Conversely, cells rendered partially deprived of MT, by transfection with a plasmid vector in which the MT-I cDNA is antisense oriented in relation to a simian virus 40 promoter, became more susceptible to the DNA-damaging action of H_2O_2. The transfected cells did not exhibit alterations of GSH, superoxide dismutase- and H_2O_2-destroying enzymes. Indirect immunofluorescence indicated that most of the MT was concentrated in the cell nucleus. Neither overexpression nor lower expression of MT resulted in differential resistance to the killing action of H_2O_2. However, the combined high nuclear concentration of MT and its excellent hydroxyl scavenger properties confer protection to DNA from hydroxyl radical attack.

INTRODUCTION

Metallothioneins (MT) are small proteins in which cysteine residues comprise 23–33% of the amino acids. Their biological role has been a matter of debate (Karin, 1985). Besides being involved in the metabolism of heavy metals, other biological functions have been considered. For instance, it has been proposed that on account of its high cysteine content, MT might play an antioxidant role in the cell (Thornalley and Vasak, 1985). Experiments *in vitro* showed that each cysteine residue in MT is 38.5-fold more effective at protecting DNA from hydroxyl radical attack than the GSH cysteine (Abel and de Ruiter, 1989).

It would be expected that cells enriched in MT would be more resistant to the killing action of oxidant species. However, recent results do not permit clear conclusions to be drawn in this regard. An earlier paper (Bakka et al., 1982) reported that cells in which MT was induced by Cd became more resistant to ionizing radiation. More recently we obtained cells rendered resistant to Cd by chronic exposure to increasing concentrations of this metal ion and found that these cells became cross-resistant to H_2O_2 (Mello-Filho et al., 1988). The high levels of MT in these cells indicated that this protein could be the source of resistance to oxidative stress. However, in ensuing work we determined that the increase in GSH content obtained by the Cd treatment was, in fact, responsible for the cross-resistance to oxidative stress (Chubatsu et al., 1992). To clarify the problem, conditions should be developed in which MT is differentially expressed without changes in the intracellular GSH level. One would expect that such a situation would occur if cells were transfected with expressing vectors containing MT genes. At least two such cases have been reported (Lohrer and Robson, 1989; Kaina et al., 1990) in which cells overexpressing MT did not become more resistant than the parental cells to the lethal effect of ionizing radiation.

An independent way of eliciting MT overexpression, without concomitant increase in GSH, is by exposure to Zn (Ochi et al., 1988). Using this approach we obtained cells that were enriched in MT but that were not cross-resistant to the killing effect of H_2O_2 (Chubatsu et al., 1992). Taken together these results indicate that MT does not contribute significantly as a protector against the lethal effect of oxidative stress.

The question then arises as to whether this conclusion can be generalized in terms of all cellular targets. Because it has been shown that MT is a protein that can be found in the nucleus (Banerjee et al., 1982; Tsujikawa et al., 1991), one might expect that the DNA-strand-breaking effect of oxidative stress could be influenced by the different levels of MT in this organelle. We set out to investigate this possibility using cells that either overexpressed or underexpressed MT in relation to the parental cells. A very sensitive and reproducible method for detecting DNA-strand breaks was employed. It was possible to show that, contrary to the cell-killing effect, the DNA-strand-breaking effect of oxidative stress was influenced by the cellular MT content.

MATERIALS AND METHODS

Cells

M8 is a clone derived from V79 Chinese hamster lung fibroblast. The cells were grown routinely in Dulbecco's modified Eagle's medium, pH 7.0, supplemented with 10% (v/v) fetal 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. Mycoplasma-free cells were used in the experiments, as determined by staining with Hoechst 33528 (Fox, 1981).

For exposure of cells to Cd^{2+} and Zn^{2+} ions, $CdSO_4$ and $ZnCl_2$ were added to the medium at the indicated concentrations.

RAG15 is a hypoxanthine-guanine phosphoribosyltransferase $(HGPRT)^-$ phenotype derived from M8 by mutagenesis with H_2O_2 and selection for resistance to 30 μg/ml of 8-azaguanine (Nassi-Calo et al., 1989).

To determine cell survival, 300 cells were plated on 35-mm diam. Petri dishes which 8 h later received the indicated Cd

Abbreviations used: MT, metallothionein; HGPRT, hypoxanthine-guanine phosphoribosyltransferase; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); SV40, simian virus 40; SOD, superoxide dismutase.

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concentrations, plus half that concentration of Zn. After 7 days of growth, the colonies were fixed with 3% (v/v) formaldehyde and stained with 0.3% Crystal Violet. Survival was scored as the ability of a cell to form a colony with at least 20 cells.

Cell extracts

Cells were trypsinized, resuspended in PBS containing 1% (v/v) fetal serum and washed three times with PBS. Lysis was performed by sequential freezing and thawing (ethanol/solid CO_2 and 37 °C water-bath) and the resulting suspension was centrifuged for 10 min at 10000 ***g***. The supernatant was used for protein determination (Hartree, 1972) and enzyme assays.

H_2O_2 consumption

Twelve hours after plating, the cells (5×10^5/dish) were washed with PBS and 5 ml of 0.1 mM H_2O_2 in PBS was added. After the indicated times at 37 °C, aliquots (100 μl) were removed to determine the H_2O_2 concentration (Cotton and Dunford, 1973).

Cell labelling and H_2O_2 treatment

Cells were labelled for 24 h in medium containing 1 μCi/ml of [^{3}H]methylthymidine (50 Ci/mmol, Amersham) after which they were trypsinized and seeded into multi-well plates (Costar, 2 cm^2/16-mm diam. well). Each well received 100000 cells which were allowed to grow for 15 h in radioisotope-free medium. At this point the medium was removed, the cells were washed with PBS and supplied with 1 ml of PBS containing the indicated concentrations of H_2O_2, which had been determined just before the experiment (Cotton and Dunford, 1973). Incubation proceeded for 30 min at 37 °C in the dark. It is important to note that the indicated H_2O_2 concentrations correspond to those at the beginning of the incubation, since cellular enzymes rapidly consume H_2O_2 from the medium (see the Results section).

DNA-strand scission

The method used is a modification of the DNA precipitation assay (Olive, 1988). After cell treatment, 300 μl of lysis buffer (10 mM Tris/10 mM EDTA/0.05 M NaOH/2% (w/v) SDS, pH 12.4) and 300 μl of 120 mM KCl were added to the well. Incubation was carried out at 65 °C for 10 min in a water-bath, after which time 400 μl of the lysate was transferred to Eppendorf tubes and kept for 5 min in an ice-bath. A DNA–protein–K–SDS complex is formed in this process, which precipitates at low temperature. Low-molecular-mass single-strand DNA was released from the bulk of the DNA during this procedure and was separated from the precipitate by centrifugation at 2500 ***g*** for 10 min at 10 °C. The supernatant was transferred to 1.5 × 2.0 cm pieces of thick filter paper; the sediment was dissolved in 100 μl of water at 65 °C and transferred to another piece of paper. These were serially washed in 5% (w/v) trichloroacetic acid, ethanol (twice) and acetone, dried and their radioactivity counted in a scintillation counter. In all plots the points represent the means of three independent determinations and the vertical bars represent the S.D.

MT determination by e.l.i.s.a.

MT-1 from rabbit liver was linked to haemocyanin, emulsified with Freund's complete adjuvant (1:1, v/v) and 50 μg of the emulsion were injected subcutaneously into mice. After 7, 14 and 28 days, 50 μg of the MT–haemocyanin were injected intraperitioneally and 10 days after the last injection the animals were bled and the antiserum titrated with goat anti-(mouse IgG), conjugated with peroxidase. Western-blot analysis revealed that the antiserum recognized both MT-I and MT-II (results not shown). E.l.i.s.a. was carried out as described previously (Knight and Sunde, 1987). The MT–antibody complex formed in the e.l.i.s.a. plate wells, previously sensitized with authentic rabbit MT-I or cell extracts, was quantified by reaction with peroxidase-conjugated goat anti-(mouse IgG) antibody. The substrate was *o*-phenylene diamine and plates were read in an e.l.i.s.a. reader (Titertek Multiskam MCC/340) at 492 nm. Preliminary assays showed that maximum sensitivity was obtained in the ranges of 1–100 ng of MT/well and 10–1000 ng of cell-extract protein/well. In these ranges good linearity was observed between A_{492} values and MT mass or between A_{492} values and protein mass in the cell extract, allowing determination of the mass of MT/mass of cellular protein ratio (results not shown).

Immunofluorescence analysis

Forty-eight hours after plating, the cells were fixed with 3.7% (v/v) formaldehyde for 5 min and permeabilized with 1% (v/v), Nonidet P40 in PBS for 15 min at room temperature. Incubation with MT antiserum, or with an antiserum which does not recognize MT, was carried out for 30 min at 37 °C, followed by addition of antibody against mouse IgG conjugated to fluorescein isothiocyanate.

GSH determination

An equal volume of 2 M $HClO_4$/4 mM EDTA was added to the cell extract and the precipitated proteins were sedimented using a minifuge for 30 min. The supernatant was neutralized with 2 M KOH/0.3 M MOPS and the $KClO_4$ was removed by centrifugation. For the spectrophotometric determination, 910 μl of the cell-extract supernatant or the GSH solution, in phosphate/EDTA buffer, were mixed with 50 μl of 4 mg/ml NADPH in 0.5% $NaHCO_3$, 20 μl of a solution of 6 units of GSH reductase/ml in phosphate/EDTA buffer and 20 μl of 1.5 mg/ml 5,5′-dithiobis(2-nitrobenzoic acid) in 0.5% $NaHCO_3$. The increase in A_{412} values under these conditions is proportional to the sum of GSH and GSSG concentrations in the cell extract (Akerboom and Sies, 1981).

Assay of superoxide dismutase (SOD) activity

SOD activity was determined from the inhibition of hydroxylamine oxidation by superoxide anion (Oyanagui, 1984).

Plasmids

pBR-MTI contains the mouse MT-I cDNA cloned on the *Eco*RI and *Hin*dIII sites of pBR322 (Durnam et al., 1980). It was removed from the PBR322 background and subcloned on the expression vector BLpSV (a kind gift from Dr. Craig Rosen, Nutley, NJ, U.S.A.). BLpSV has the simian virus 40 (SV40) early promoter, a polyadenylation signal and a multiple cloning site on a Bluescript SK background. Cutting with *Eco*RI and *Hin*dIII imposes the ligation of the MT-I cDNA in the anti-sense orientation; this was confirmed by restriction analysis. The resulting plasmid was termed BLpSV-MTas. For cell-transfection purposes the plasmid pSV2-gpt (Mulligan and Berg, 1980) was employed; it has the bacterial analogue of the mammalian HGPRT gene, under control of the SV40 promoter.

Cell transfection

The calcium phosphate method was employed (Ausubel et al., 1989). RAG15 cells were co-transfected with 1 μg of pSV2-gpt and 10 μg of BLpSV-MT. Selection of *gpt*$^+$ cells was performed in HAT medium (1×10^{-4} M hypoxanthine/4×10^{-7} M aminopterin/1.6×10^{-5} M thymidine). Two HAT-resistant clones were derived with the antisense construction, GASMT1 and GASMT2. As a control, the plasmid pBR-MTI was substituted for BLpSV-MT and one of the resulting clones, GPBMTI, was used in the experiments.

Northern-blot analysis

DNA- and Northern-blot analysis were performed essentially as described previously (Sambrook et al., 1989). RNA extraction was carried out as described by Birnboim (1988).

RESULTS

Strand scissions in cells overexpressing MT

Cells were either exposed to Cd plus Zn for 48 h or to Zn alone for 16 h. In the former case MT and GSH levels increased 1.8-fold and 2.4-fold respectively. Exposure to Zn alone induced a 2.7-fold increase in MT, whereas the GSH content remained essentially unaltered, in agreement with previous work (Ochi et al., 1988; Chubatsu et al., 1992). The results in Figure 1 show that the parental M8 cells were significantly more sensitive to H_2O_2 in terms of production of DNA-strand scission. This method has been well characterized and is based on the fact that the amount of radioactive DNA released from a SDS–K precipitate increases with the increase in the number of DNA scissions (Olive, 1988; Martins et al., 1991). M8 cells treated with Zn only exhibited an intermediate sensitivity, which should be ascribed to the higher MT content. M8 cells treated with Cd and Zn were the most resistant, because they exhibit higher MT and GSH contents, both contributing to the antioxidant protection.

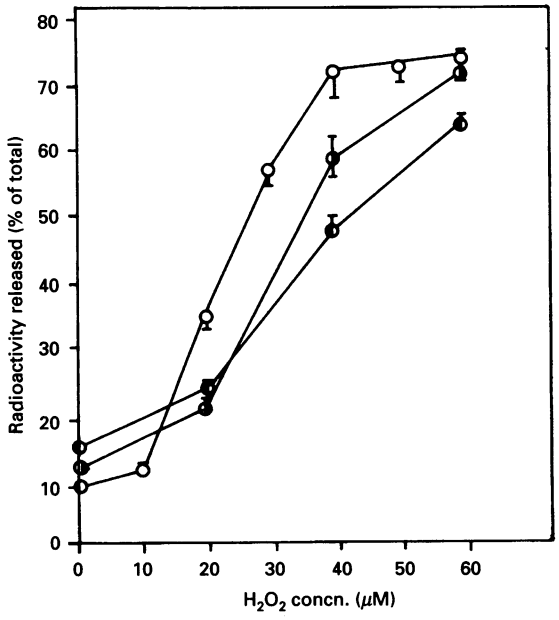

Figure 1 DNA-strand scissions produced by H_2O_2

The bars represent the S.D. for three independent determinations. ○, M8 cells; ◑, M8 cells cultured in the presence of 100 μM Zn for 16 h; ◐, M8 cells cultured in the presence of 100 μM Cd and 50 μM Zn.

The major differences in sensitivities were observed in an intermediate range of H_2O_2 concentrations. This is due to the fact that above certain levels of strand scission there is a tendency for scission to plateau because residual DNA in the SDS–K^+ gel is not released in the supernatant (Martins et al., 1991).

Obtaining cells that underexpress MT

We set out to obtain cells that underexpress MT using the antisense mRNA approach. For this purpose the BLpSV-MTas plasmid, containing the mouse MT-I cDNA antisense-oriented in relation to the SV40 promoter, was used to transfect RAG15 cells. This is a cell line derived from M8 by mutagenesis with H_2O_2 and selection with 8-azaguanine and that lacks the *HGPRT* gene. This cell line is convenient to use because it permits a co-transfection with BLpSV-MTas and pSV2-gpt plasmids (Mulligan and Berg, 1980) followed by selection of the resistant cells in HAT medium. A control co-transfection was also carried out with pSV2-gpt plus pBR-MTI; this latter plasmid contains the MT-I cDNA in the pBR322 background and is not under any promoter control. The former co-transfection generated two clones named GASMT1 and GASMT2. The co-transfection with the control pBR-MT1 plasmid generated a clone named GPBMT1.

The cell survival experiment illustrated in Figure 2 showed that the RAG15 and the GPBMT1 cells exhibited essentially the same resistance to Cd, indicating that the introduction of a non-expressable MT cDNA plasmid into RAG15 cells does not alter their capacity to deal with the heavy metal. On the other hand, the clones GASMT1 and GASMT2, transfected with the antisense construction, were clearly more sensitive to Cd. This was certainly due to a decrease in the levels of MT in these cells, as

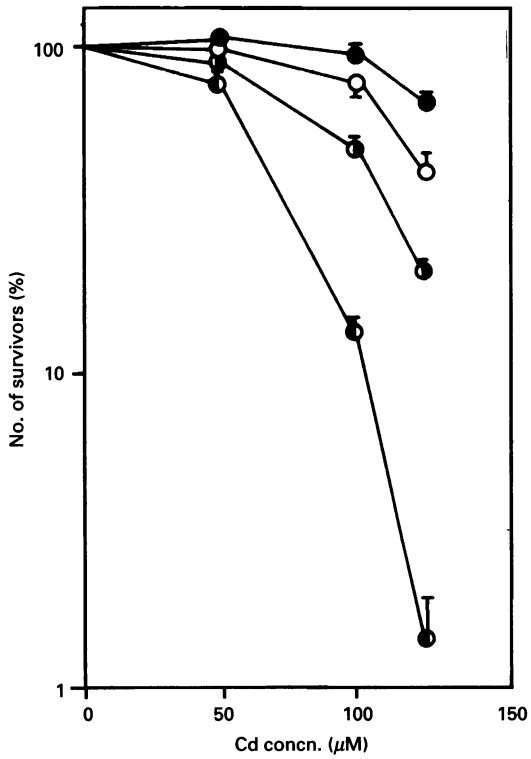

Figure 2 Cell killing by Cd/Zn

The concentration of Zn was half that of Cd. Key to symbols: ○, RAG15; ●, GPBMT1; ◑, GASMT1; ◐, GASMT2. The bars indicate the S.D. for three independent determinations.

Table 1 MT content in transfected cells

MT determinations (±S.D.) were performed by e.l.i.s.a. MT content in M8 cells is 3×10^{-12} g/cell (Chubatsu et al., 1992).

Cells	MT content relative to M8 cells
M8	1.00
RAG15	0.94±0.06
GPBMT1	1.00±0.10
GASMT1	0.68±0.01
GASMT2	0.70±0.09

Table 2 GSH content and SOD activity in transfected cells

GSH is expressed as ng/μg of protein and SOD in terms of units/mg of protein. The values correspond to the mean±S.D. for the number of determinations indicated in parentheses.

Cells	GSH content (ng/μg of protein)	SOD activity (unit/mg of protein)
M8	4.8±0.7 (2)	3.5±0.8 (4)
RAG15	4.8±0.2 (4)	3.5±1.3 (4)
GPBMT1	5.2±0.5 (4)	4.0±0.5 (4)
GASMT1	4.6±0.6 (6)	5.0±0.7 (4)
GASMT2	5.2±0.5 (4)	4.5±1.2 (4)

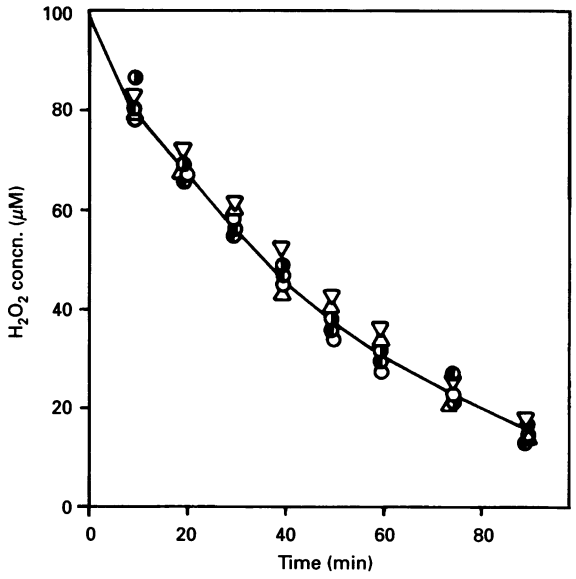

Figure 3 Decomposition of H_2O_2 by the transfected cells

Key to symbols: ○, M8; ◑, RAG15; ◐, GPBMT1; △, GASMT1; ▽, GASMT2.

shown in the e.l.i.s.a. experiment shown in Table 1. These levels were maintained unaltered in M8, RAG15 and GPBMT1 cells, whereas a significant decrease of 30% was observed in GASMT1 and GASMT2 cells. Given the fact that the reduction in the MT levels in these two cells was the same, it is not clear why the killing by Cd is more pronounced in GASMT2 than in GASMT1.

To rule out the possibility that the intracellular levels of other antioxidants have changed in these transfectants, we measured GSH and SOD levels. The results (Table 2) clearly indicated that no major change in these parameters had occurred for all the

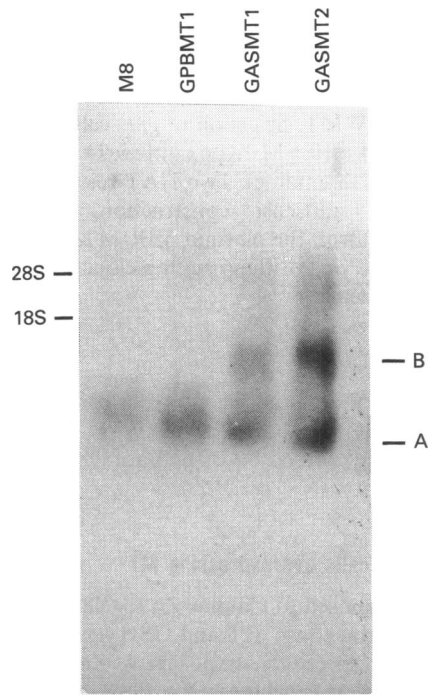

Figure 4 Northern blot analysis

The radioactive probe was ^{32}P-labelled mouse MTI cDNA. A corresponds to the endogenous MTI mRNA and B corresponds to the RNAs transcribed from the transfected antisense-oriented MTI DNA.

cells investigated. The ability of these cells, which express a combined activity of catalase and glutathione peroxidase, to destroy H_2O_2 added to the medium was also identical (Figure 3).

Experiments investigating survival after exposure to H_2O_2 revealed that GASMT1 and GASMT2 exhibited the same killing rate, which did not differ from those of M8, RAG15 and GPBMT1 cells (results not shown). This is in agreement with previous results (Chubatsu et al., 1992), corroborating that MT alone does not confer extra protection against the lethal action of oxidative stress.

Transcription of the antisense mRNA

To determine the pattern of MT-RNA transcription in the transfected cells the Northern-blot analysis shown in Figure 4 was carried out. The results showed that, in addition to the endogenous MT-mRNA observed in RAG15 and GPBMT1 cells, the mouse MT-cDNA probe revealed another band of slightly higher molecular mass in the GASMT1 and GASMT2 cells. These bands correspond presumably to the antisense MT-mRNA, giving the construction that was used to transfect these cells.

DNA scission in cells underexpressing MT

The experiment shown in Figure 5 clearly indicated that the GASMT1 and GASMT2 cells were more sensitive to the DNA-strand-breaking action of H_2O_2 than were the non-transfected M8 and RAG15 cells or the GPBMT1 cell, transfected with a non-expressable vector. As in the experiment investigating resistance to Cd, the difference between GASMT1 and GASMT2 in withstanding oxidative attack is not clear, given the fact that

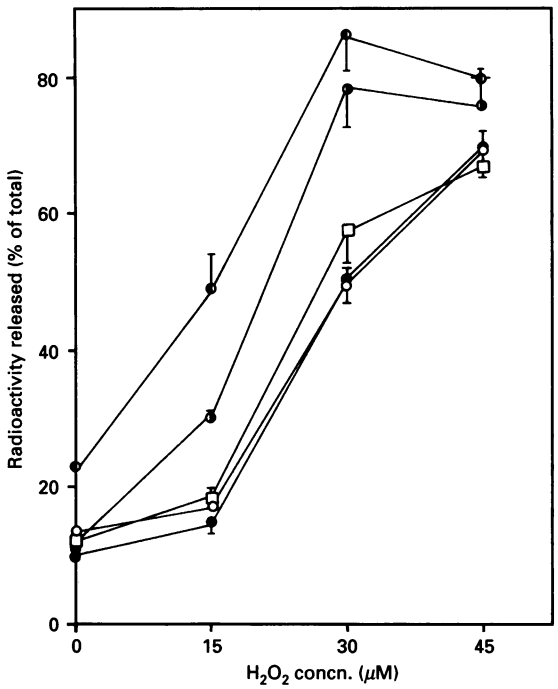

Figure 5 DNA-strand scissions produced by H_2O_2 in transfected cells

Key to symbols: ○, M8; ●, RAG15; □, GPBMT1; ◑, GASMT1; ◐, GASMT2.

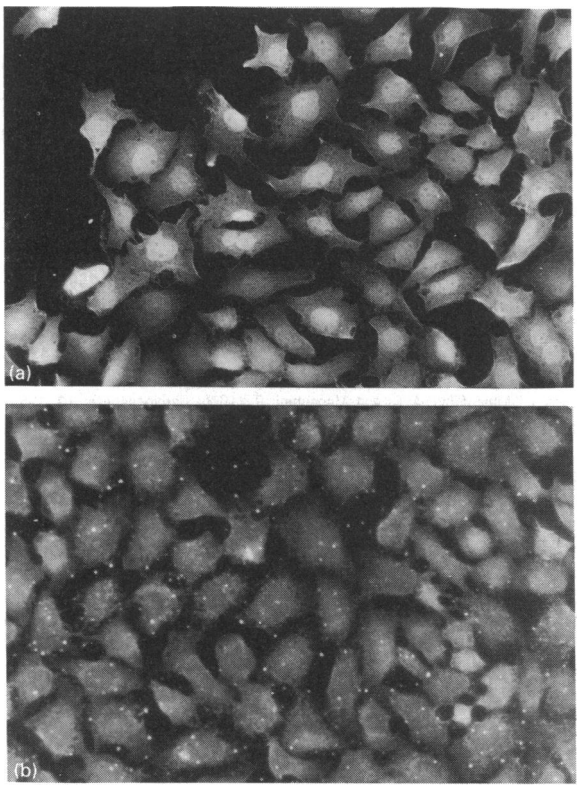

Figure 6 Indirect immunofluorescence of MT

Cells were chronically exposed to Cd (Mello-Filho et al., 1988). In (**a**) immunofluorescence was obtained with an antiserum raised against MT. (**b**) shows a control with an antiserum that does not recognize MT.

both exhibit the same reduction in MT content. Here again it should be emphasized that the intermediate range of H_2O_2 concentration is the one that should be considered, since at high concentrations a limiting value of DNA solubilization is attained, irrespective of additional DNA scissions.

MT in the nucleus

One possible mechanism of MT protection against H_2O_2-produced strand scission is through its strong hydroxyl radical scavenger effect (Thornalley and Vasak, 1985). If this were the case one should expect that MT exerted its effect in the nucleus, in the DNA vicinity. This is because the hydroxyl radical travels on average 1.5 nm before reacting with DNA in the case of H_2O_2-produced DNA-strand scission (Ward et al., 1985). Therefore a scavenging effect would only be effective if the agent were very close to the site of hydroxyl radical generation. To examine the presence of MT in the nucleus an indirect immunofluorescence technique was employed. Figure 6(a) shows the results with M8 cells chronically exposed to Cd (Mello-Filho et al., 1988), but the same pattern was obtained with untreated M8 cells, although with lower fluorescence intensity. It is quite clear that MT is abundant in the nucleus. It is also evident that not all the nuclei show fluorescence by this assay. These results are similar to those obtained with rat hepatocytes (Tsujikawa et al., 1991) in which case the presence of MT in the nuclei was only detected in S-phase cells.

DISCUSSION

The role of MT in protecting the cells against the toxic effects of agents other than heavy metals has been in debate in recent years. Because of its peculiar structure, characterized by a large content of thiol groups, MT is endowed with strong antioxidant and nucleophilic properties. On account of that it is possible that MT overexpression constitutes a mechanism of acquired drug resistance to alkylating compounds (Kelley et al., 1988; Lohrer and Robson, 1989; Kaina et al., 1990), although this possibility has been disputed (Schilder et al., 1990).

Another possible role for MT is that it constitutes an important defence against oxidative stress. In fact, this protein seems to have an excellent capacity for scavenging hydroxyl radicals (Thornalley and Vasak, 1985; Abel and de Ruiter, 1989). However, controversy has arisen with regard to the physiological antioxidant role of MT. Cells engineered by gene transfection to overexpress MT did not exhibit extra resistance to γ rays (Lohrer and Robson, 1989; Kaina et al., 1990) or to bleomycin (Lohrer and Robson, 1989). This is at odds with the observation that cells induced to overexpress MT by previous exposure to Cd became cross-resistant to oxidative stress (Bakka et al., 1982; Mello-Filho et al., 1988). However, exposure to Cd induces an increase of the GSH level as well (Seagrave et al., 1983), and this was shown to be the basis for the cross-resistance to oxidative stress (Chubatsu et al., 1992). In fact, exposure to Zn led to overexpression of MT without concomitant increase of the GSH level (Ochi et al., 1988), and in this case the cells did not become cross-resistant to oxidative stress (Chubatsu et al., 1992). Therefore, the weight of evidence seems to favour the hypothesis that MT does not protect significantly against the lethal action of oxidative stress *in vivo*.

Nevertheless, a cautionary word seems to be appropriate against definite conclusions since it has been shown recently that injection in rats of several agents that produce oxidative stress led to an increase in the MT level in several organs (Bauman et al., 1991). It is possible that MT plays a protective role, in specific

cellular loci, against oxidative damage. One such locus could be the nucleus. In fact, the presence of MT in the nucleus has been reported (Banerjee et al., 1982; Tsujikawa et al., 1991) and was confirmed in the present work. It is conceivable that DNA could be protected by MT from oxidative damage. We obtained cells enriched in, or partially depleted of, MT by exposure to Zn and by the antisense mRNA approach respectively. In either case there was no change in other antioxidant cellular components like GSH, SOD, catalase or glutathione peroxidase. Very clearly, cells enriched in MT suffered less H_2O_2-produced DNA-strand scission than the parental cells, and the opposite was observed for cells partially depleted in MT. Zinc has been suggested to protect DNA directly by displacing Fe and Cu from sites where they mediate production of oxidative damage (Korbashi et al., 1989). However, it seems that in the present case the protection is through the induction of MT. In fact, the incubation time in the presence of Zn required for the protection to be observed is equivalent to the time necessary for MT to start to be accumulated (Chubatsu et al., 1992).

The mechanism involved in the protective effect has not been established. The evidence so far obtained indicates that H_2O_2 reacts with Fe(II) bound to DNA to generate hydroxyl radicals that are very close to the DNA target (Mello-Filho and Meneghini, 1984, 1985). Protection by hydroxyl radical scavenger under this circumstance is difficult and can only be attained by species that have a high rate constant towards the hydroxyl radical and that reach significant concentrations in the nucleus. The former requirement is totally met by MT, which has been shown to protect DNA *in vitro* from hydroxyl radical attack with a 800-fold higher molar efficiency than GSH (Abel and de Ruiter, 1989).

We have determined that M8 cells have 3.0×10^{-12} g of MT/cell (Chubatsu et al., 1992). Immunofluorescence indicates that a great part of the MT is concentrated in the nucleus. If we consider that 50% of the MT is in the nucleus its concentration there will be 360 mM. If we recall that in order to protect DNA from hydroxyl radical attack, extracellular scavengers should be added in the 200–600 mM range (Mello-Filho and Meneghini, 1984; Ward et al., 1985), it is expected that MT, at the concentrations reached in the nucleus, does afford protection to DNA. Another thiol compound that has been reported to be concentrated in the nucleus is GSH (Bellomo et al., 1992). Its concentration reaches 19 mM in mouse hepatocyte nucleus, which is, however, well below the MT concentration found in the M8 nucleus. This, added to the fact that MT is 800 times more efficient than GSH in protecting DNA from hydroxyl radical attack (Abel and de Ruiter, 1989), indicates that MT is a more important nuclear antioxidant than GSH. However, other mechanisms of protection are not precluded. The ability of MT to complex Fe has been reported (Thomas et al., 1986; Good and Vasak, 1986) and it is possible that the protein removes Fe from sites where its efficiency at producing DNA-damaging hydroxyl radicals is high. However, Cu rather than Fe is usually found associated to MT *in vivo*. It is interesting to speculate that the high MT concentration in the nucleus may provide protection against the Cu-mediated oxidative reactions, considering that this ion is much more effective than Fe as a Fenton reagent.

The fact that MT is less efficient at protecting from lethal events than from DNA damage raises again the question of how important DNA is as a target for lethal events. It seems clear that single-strand scissions *per se* are not relevant in terms of DNA lethality (Ward et al., 1985). Probably, other cellular hydroxyl radical targets, which are less accessible to MT, are more important in terms of cell killing. However, because hydroxyl radicals produce several mutagenic base damages, in addition to strand scission (Aruoma et al., 1989), MT might be important as an endogenous anti-mutagenic agent.

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REFERENCES

Abel, J. and de Ruiter, N. (1989) Toxicol. Lett. **47**, 191–196
Akerboom, T. P. M. and Sies, H. (1981) Methods Enzymol. (Jacob, W. B., ed.), vol. 77, pp. 373–382, Academic Press, New York
Aruoma, O. I., Halliwell, B. and Dizdaroglu, M. (1989) J. Biol. Chem. **264**, 13024–13028
Ausubel, F. M., Brent, R., Kingnston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A. and Struhl, K. (1989) Current Protocols in Molecular Biology, Wiley Interscience, New York
Bakka, A., Johnsen, A. S., Endresen, L. and Rugstad, H. E. (1982) Experientia **38**, 381–383
Banerjee, D., Onosaka, S. and Cherian, M. G. (1982) Toxicology **24**, 95–105
Bauman, J. W., Liu, J., Liu, Y. P. and Klaassen, C. D. (1991) Toxicol. Appl. Pharmacol. **110**, 347–354
Bellomo, G., Vairetti, M., Stivala, L., Mirabelli, F., Richelmi, P. and Orrenius, S. (1992) Proc. Natl. Acad. Sci. U.S.A. **89**, 4412–4416
Birnboim, H. C. (1988) Nucleic Acid Res. **16**, 1487–1497
Chubatsu, L. S., Gennari, M. and Meneghini, R. (1992) Chem.-Biol. Interact. **82**, 99–110
Cotton, M. L. and Dunford, H. B. (1973) Can. J. Chem. **51**, 582–587
Durnam, D. M., Perrin, F., Gannon, F. and Palmiter, R. D. (1980) Proc. Natl. Acad. Sci. U.S.A. **77**, 6511–6515
Fox, M. (1981) in DNA Repair (Friedberg, E. C. and Hanawalt, P. C., eds.), pp. 523–543, Marcel Dekker Inc., New York
Good, M. and Vasak, M. (1986) Biochemistry **25**, 8353–8356
Hartree, E. F. (1972) Anal. Biochem. **48**, 422–427
Kaina, B., Loher, H., Karin, M. and Herrlich, P. (1990) Proc. Natl. Acad. Sci. U.S.A. **87**, 2710–2714
Karin, M. (1985) Cell **41**, 9–10
Kelley, S. L., Basu, A., Teicher, B. A., Hacker, M. P., Hamer, D. H. and Lazo, J. S. (1988) Science **241**, 1813–1815
Knight, S. A. B. and Sunde, R. A. (1987) J. Nutr. **117**, 731–738
Korbashi, P., Katzhendler, J., Saltman, P. and Chevion, M. (1989) J. Biol. Chem. **264**, 8479–8482
Lohrer, H. and Robson, T. (1989) Carcinogenesis **10**, 2279–2284
Martins, E. A. L., Chubatsu, L. S. and Meneghini, R. (1991) Mutat. Res. **250**, 95–101
Mello-Filho, A. C. and Meneghini, R. (1984) Biochim. Biophys. Acta **781**, 56–63
Mello-Filho, A. C. and Meneghini, R. (1985) Biochim. Biophys. Acta **847**, 82–89
Mello-Filho, A. C., Chubatsu, L. S. and Meneghini, R. (1988) Biochem. J. **256**, 475–479
Mulligan, R. C. and Berg, P. (1980) Science **209**, 1422–1427
Nassi-Calo, L., Mello-Filho, A. C. and Meneghini, R. (1989) Carcinogenesis **10**, 1055–1057
Ochi, T., Otsuka, F., Takahashi, K. and Ohsawa, M. (1988) Chem.-Biol. Interact. **65**, 1–14
Olive, P. L. (1988) Environ. Mol. Mutagen **11**, 487–495
Oyanagui, Y. (1984) Anal. Biochem. **142**, 290–296
Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, New York
Schilder, R. J., Hall, L., Monks, A., Handel, L. M., Fornace, Jr., A. J., Ozols, R. F., Fojo, A. T. and Hamilton, T. C. (1990) Int. J. Cancer **45**, 416–422
Seagrave, J., Hildebrand, C. E. and Enger, M. D. (1983) Toxicology **29**, 101–107
Thomas, J. P., Bachowski, G. J. and Girotti, A. W. (1986) Biochim. Biophys. Acta **884**, 448–461
Thornalley, P. J. and Vasak, M. (1985) Biochim. Biophys. Acta **827**, 36–44
Tsujikawa, K., Imai, T., Kakutani, M., Kayamori, Y., Mimura, T., Otaki, N., Kimura, M., Fukuyama, R. and Shimizu, N. (1991) FEBS Lett. **283**, 239–242
Ward, J. F., Blakely, W. F. and Joner, E. I. (1985) Radiat. Res. **103**, 383–392

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