A Role for Metallothionein and Zinc in Spontaneous Mutagenesis

Ekaterina I. Goncharova and Toby G. Rossman

ABSTRACT

G12, a transgenic Chinese hamster V79 cell derivative which contains a single copy of the Escherichia coli gpt gene as a target for mutagenesis, has little constitutive metallothionein (MT) expression. It was transfected with a vector containing the mouse MT-I gene, and MT-I-overproducing lines were isolated. MT-I transfectants had lower spontaneous mutation frequencies compared with the G12 parental cell line. Mutagenesis by alkylating agents was unchanged. MT expression in G12 and MT transfectants could be modulated by exposure to Zn(II) or Cd(II). The spontaneous mutation frequencies in Zn(II)- and Cd(II)-treated cells was inversely related to MT expression. In G12 cells grown in concentrations of Zn(II) up to 12 μM, a significant dose-dependent increase in spontaneous mutagenesis was observed. At higher (but subtoxic) concentrations in which endogenous MT was induced, a dramatic decrease in spontaneous mutagenesis was observed. In contrast, MT-I transfectants exhibited much lower spontaneous mutagenesis after growth in all concentrations of Zn(II). These data demonstrate a possible role for MT in modulating spontaneous mutagenesis and point to a role for Zn(II) in contributing to spontaneous mutagenesis. Because there is variability in human MT expression, low MT expression might be a risk factor for cancer.

INTRODUCTION

MTs are low molecular weight sulfhydryl-rich proteins which are present throughout the phylogenetic spectrum (1). Cysteines account for about one-third of the amino acids in MT. This unique structure allows binding of metals. Metals which bind to MT under physiological conditions include the nutritionally essential trace elements zinc and copper, as well as the potentially toxic element cadmium (2).

The biological functions of MTs are not clearly resolved. Since their discovery, it has been suggested that MTs play a crucial role in detoxification of heavy metals and perhaps in the storage of metal ions and the regulation of cellular Zn(II) and Cu(II) metabolism. Zinc is an essential participant in a number of DNA and RNA polymerases and serves as a structural feature of the zinc finger domains in at least 300 essential participant in a number of DNA and RNA polymerases and serves as a structural feature of the zinc finger domains in at least 300 enzymes. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

MTs are low molecular weight sulfhydryl-rich proteins which are present throughout the phylogenetic spectrum (1). Cysteines account for about one-third of the amino acids in MT. This unique structure allows binding of metals. Metals which bind to MT under physiological conditions include the nutritionally essential trace elements zinc and copper, as well as the potentially toxic element cadmium (2).

The biological functions of MTs are not clearly resolved. Since their discovery, it has been suggested that MTs play a crucial role in detoxification of heavy metals and perhaps in the storage of metal ions and the regulation of cellular Zn(II) and Cu(II) metabolism. Zinc is an essential participant in a number of DNA and RNA polymerases and serves as a structural feature of the zinc finger domains in at least 300 enzymes. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Investigations of a possible role for MT in carcinogenesis have only recently begun. The best case that can be made for a protective role for MT occurs in cadmium carcinogenesis. Rat, mouse, and monkey testes, rat ventral prostate, and hamster ovary are all known to be targets of cadmium carcinogenesis. These tissues appear to be deficient in MT (9). Elevated levels of MT have been found in malignant human liver tumors (10) and in murine tumors derived from transformed 3T3 cells in several tumors of nonhematopoietic origin such as testicular carcinoma (11). High levels of MT were detected in papillomas and carcinomas induced in mouse skin initiated with 7,12-dimethylbenz(a)anthracene and promoted with phorbol ester (12).

An abundance of recent data on molecular changes in carcinogenesis provides strong evidence that mutational events play an important role in the malignant process (13). This laboratory has been involved in elucidating the genetic changes induced by carcinogenic metal compounds (14–16). Because MT expression is sometimes downregulated in permanent cell lines (1), no metal mutagenesis studies have been performed in cells producing high levels of MT. To gain an understanding of the possible role of MT in metal mutagenesis, we have created MT-expressing cell lines using the transgenic cell line G12, which was previously developed in this laboratory (17). The G12 line is derived from Chinese hamster V79 cells and contains a single copy of the Escherichia coli gpt gene as a target for mutagenesis. This target is sensitive to genetic changes arising from a variety of promutagenic lesions. Cells with mutations at the gpt locus are unable to grow in HAT medium. Growth in HAT thus allows for selection against any preexisting mutants at the start of an experiment. Like many other permanent lines, G12 cells express only a small constitutive level of MT. We have further engineered these cells by transfection with a vector containing the mouse MT-I gene.

We report here that MT-I transfectants have lower spontaneous mutation frequencies compared with the G12 parental cell line, and that the spontaneous mutation frequency is inversely related to the level of MT expression. In addition, we provide evidence that at least some fraction of spontaneous mutagenesis may be due to the mutagenic action of Zn(II) in the absence of sufficient MT.

MATERIALS AND METHODS

Cell Culture. The cell lines used were V79 and G12, a gpt transfectant of Chinese hamster V79 cells (17). The cells were grown in Ham's F-12 medium, supplemented with 5% fetal bovine serum (GIBCO, Grand Island, NY) and 50 μg/ml penicillin-streptomycin (GIBCO) at 37°C in an atmosphere of 5% CO2 and 95% air. The cells have a rapid growth rate (doubling time, 12–16 h) and high cloning efficiency.

Cotransfection of pBPV-MT-Xho and pSV2neo in G12 Cells. G12 cells were cotransfected with 10 μg of pBPV-MT-Xho and 2 μg of pSV2neo (American Type Culture Collection, Rockville, MD) using the calcium phosphate DNA precipitation method (18), followed by glycerol treatment (15% glycerol-15 mM 4(2-hydroxyethyl)-1-piperazineethanesulfonic acid for 3 min.) pBPV-MT-Xho was kindly provided by Dr. D. Hamer (NIH, Bethesda, MD). pBPV-MT-Xho contains the mouse MT-I gene (19) cloned in a bovine papillomavirus vector (20). After overnight incubation, cells were washed with Earle's balanced salt solution (GIBCO) and the medium was changed. Cells were split 24 h later and plated in selective medium containing 700 μg/ml of genetin (Sigma Chemical Co., St. Louis, MO). The medium was changed after one week. Two weeks later, geneticin-resistant clones were isolated.

Neutral Red Toxicity Assay. For Neutral Red toxicity assay (21), cells were seeded at a concentration of 104/well in a microtiter plate and continuously exposed to various concentrations of cadmium chloride for 48 h. Cells were then exposed to 50 μg/ml of Neutral Red (Fisher Scientific, Fair Lawn, NJ) for 3 h. Medium was then removed and cells were rapidly washed with a fixative (1% formaldehyde-1% CaCl2), followed by addition of 0.2 ml of a
mixture of 1% acetic acid-50% ethanol to extract the dye from the cells. The absorbance was monitored on a microtitr e plate reader at 550 nm.

Mutagenesis Assays. Mutagenesis by alkylating agents at the gpt locus was performed as described previously (17). MNNG and EMS were purchased from Sigma. Exposure of cells to MNNG was for 15 min in Earle’s balanced salt solution (GIBCO). Exposure to EMS was overnight in medium.

Induced mutagenesis assay has been described previously for the transgenic G12 cells (17). For spontaneous mutations, the cells were grown in the presence of preexisting mutants by exposure to HAT (0.2 mM hypoxanthine-1.0 mM aminopterin-0.1 mM thymidine) for 1 week. Mutants were then allowed to accumulate in normal medium for 6 days, after which 10 dishes were reseeded at a density of 2 x 10^6 cells/100-mm dish into selection medium containing 6-thioguanine (10 mg/ml). The reseeding plating efficiency in nonselective medium was also determined for use in mutant fraction calculations. The selection medium was replenished after 1 week and mutant colonies were stained and scored after 10-13 days. The mutant fraction was calculated by dividing the number of mutant colonies by the number of cells seeded times the plating efficiency of the cells. SEs were calculated based on 10 samples for each point. It should be noted that results are reported as mutant fraction, i.e., mutants/10^6 (clonable) survivors. The spontaneous mutation fraction can vary from experiment to experiment because it is not possible to control precisely for the number of generations of growth before mutant selection. We are presently working on a mathematical approach to derive mutation rate from mutant fraction, based on knowing the number of generations undergone. All experiments reported here were repeated at least once, and the shapes of the dose-response curves were reproducible.

RNA Isolation and Northern Analysis. Total RNA was isolated using RNAsol (Biotechx Laboratories, Inc., Houston, TX) according to the manufacturer’s directions. Dot-blot arrays were prepared using multiple filtration manifold (Schleicher and Schuell, Keene, NH) on Nytran nylon membranes. For Northern hybridization, RNA (15 μg) was subjected to electrophoresis in a 1% agarose-2.2% formaldehyde gel and transferred to nylon membrane (Schleicher and Schuell) in 20X standard saline citrate buffer (0.15 M NaCl, 0.15 M sodium citrate, pH 7.0) overnight. The metallothionein hybridization probe was the gel-purified insert from EcoRI-digested plasmid pBPV-MT-Xho in 1% agarose gel. The insert was extracted with Geneclean (Bio 101, Inc., La Jolla, CA). The probe was labeled with [32P]dCTP using random primers kit (United States Biochemicals, Cleveland, OH). Northern blots were processed for hybridization in the same way, as recommended by membrane manufacturer. Hybridization with β-actin probe was carried out to equalize the loading of RNA.

RESULTS

Isolation and Characterization of MT-I Transfectants. G12 cells were cotransfected with pBPV-MT-Xho as described in “Materials and Methods.” A number of geneticin resistant clones were isolated and examined for their sensitivity to Cd(II). Fig. 1 shows that cells from four representative clones showed significant resistance to CdCl₂ cytotoxicity compared with G12 cells. MTI-2, which has the highest resistance to Cd(II), was selected for further study. The levels of MT expression in G12 cells and in a number of MT-I transfectants were determined by Northern analysis (Fig. 2). Constitutive MT expression was not detectable in G12 cells (Fig. 2, Lane 1), whereas the transfectants had considerable basal levels of expression (Fig. 2, Lanes 2–6). A subclone, MT1–2A, with even higher resistance to Cd(II), was isolated from MT1–2 by selection in a higher concentration (50 μM) of CdCl₂ (Fig. 2, Lane 5). These cells also showed a much higher level of MT mRNA compared with MT1–2 (Fig. 2, Lane 3). It was also possible to further modulate the level of MT expression in MT1–2A cells by continuous exposure to cadmium chloride (Fig. 2, Lane 6).

Effect of MT Expression on Spontaneous Mutagenesis. While using the MT1–2 cell line to study metal mutagenesis, it became apparent that its background (spontaneous) mutant fraction was much lower than that of the parental G12 line. Spontaneous mutagenesis was also decreased in 5 other MT-transfectant lines tested. To determine whether MT expression was affecting spontaneous mutagenesis, the mutant fraction was measured in G12 cells and in transfectants in which the MT content was modulated by exposure to Cd(II) or Zn(II) (Table 1). After removal of preexisting mutants by growing cells in...
Table 1  

<table>
<thead>
<tr>
<th>Cells</th>
<th>Conditionsa</th>
<th>Mutant fraction (X 10^-6)</th>
<th>Cd(II) LD50b (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>G12</td>
<td>Uninduced</td>
<td>37.9</td>
<td>35</td>
</tr>
<tr>
<td>MT1-2</td>
<td>Uninduced</td>
<td>21.6</td>
<td>66</td>
</tr>
<tr>
<td>MT1-2A</td>
<td>Selected in Cd; Cd removed 6 wk</td>
<td>12.5</td>
<td>73</td>
</tr>
<tr>
<td>MT1-2A</td>
<td>Selected in Cd; Cd removed 3 wk</td>
<td>9.0</td>
<td>83</td>
</tr>
<tr>
<td>MT1-2A</td>
<td>Selected in Cd; Cd removed 6 wk, 24 hrs in ZnCl2 (120 µM)</td>
<td>4.2</td>
<td>Not determined</td>
</tr>
</tbody>
</table>

a Uninduced refers to cells grown in normal medium without additional Zn(II) or Cd(II) for at least 12 weeks. MT1-2A cells were maintained in 50 µM CdCl2 grown in the absence of CdCl2 for 3 weeks, 6 weeks, or as indicated. 
b Sensitivity to CdCl2 was estimated at the time of mutant selection. LD50, concentration of CdCl2 resulting in 50% survival as determined in the Neutral Red assay. 
c MT1-2A cells were selected from MT1-2 cells grown for a week in the presence of 50 µM CdCl2. 
d Cd, cadmium.

Fig. 3. Fluctuation analysis of spontaneous mutation frequencies of G12 and MT1–2A. The fluctuation test was performed as described in “Materials and Methods”.

HAT medium for 1 week, mutations were allowed to accumulate for another week. As expected, G12 cells showed the highest spontaneous mutant fraction (Table 1). In the transfectants, decreased sensitivity to CdCl2 cytotoxicity at the time of mutant selection was associated with a lower spontaneous mutant fraction. Thus, in the same cell line, the inverse relationship between spontaneous mutability and presumptive MT content was demonstrated.

In order to measure the spontaneous mutagenesis in a more accurate manner, a variation of the fluctuation test was performed (see “Materials and Methods”). There was no difference in the growth rates of G12 and MT1–2A cells. The distribution of mutant fractions in each population is shown in Fig. 3. Many of the MT1–2A populations accumulated no mutants (<0.5 x 10^-6), whereas all G12 populations acquired mutants. G12 populations with the highest mutant fractions (representing the populations which sustained early mutations) contained a larger fraction of mutants than the comparable population of MT1–2A cells. The mutant fraction for all populations combined was 27.6 x 10^-6 for G12 and 8.78 x 10^-6 for MT1-2. The value for the G12 cells agrees remarkably well with the historic value of about 30 x 10^-6, calculated from over 100 experiments (23).

The effect of strong monofunctional alkylating agents was examined in G12 and in MT1–2A cells. No differences were observed in survival or in mutability between these cell lines after treatment with MNNG or EMS (Fig. 4). 

Effect of Zn(II) on Spontaneous Mutagenesis. Both Zn(II) and Cd(II) are inducers of MT. Since zinc is an essential metal and is also contained in many food supplements, it was of interest to assay the
METALLOTHIONEIN AND ZINC IN SPONTANEOUS MUTAGENESIS

mutant fraction after growth in media containing various concentrations of zinc. G12 and MT1–2A cells were grown in HAT medium containing various nontoxic concentrations of ZnCl₂ for 1 week, reseeded in growth medium in the same concentrations of ZnCl₂, grown for a second week, and mutant fractions were measured (Fig. 5A). The growth rates were similar for all zinc concentrations used. Tissue culture medium itself contains approximately 2 μM Zn(II). As the Zn(II) concentration increased to 12 μM, there was a dramatic increase in the mutant fraction in G12 cells. However, at higher concentrations the mutant fraction decreased, finally reaching a level 10 times lower than that in normal medium. In contrast, MT1–2A cells showed a consistently low mutant fraction in all concentrations of Zn(II). Northern blot analysis revealed that after growth in 37 μM Zn(II), G12 cells contained MT mRNA at a level which was higher than that in uninduced G12 cells but much lower than the basal level in MT-I transfectants (Fig. 5B). Increased levels of MT expression were also observed in MT1–2A grown in 62 μM Zn(II) (Fig. 5B). To confirm that MT protein levels reflect MT mRNA levels, indirect immunofluorescence using a mouse anti-MT monoclonal antibody (Zymed Laboratories, Inc., San Francisco, CA) was performed. Cells grown in 2 μM Zn(II) (normal growth medium) or 12 μM Zn(II) showed barely detectable staining. In contrast, intensive staining of both nucleus and cytoplasm was observed in cells grown in 37 and 47 μM Zn(II) (data not shown). Thus, growth of G12 cells in elevated but nontoxic concentration of Zn(II) led to a considerable increase in the mutant fraction, but at still higher concentrations (still below toxic levels); when MT expression was induced, a dramatic decrease in the mutant fraction was observed. In MT1–2A cells, which maintain a high level of MT expression even in low concentrations of Zn(II), the spontaneous mutant fraction was similar and low at all Zn(II) concentrations.

In both G12 and MT1–2A cells, spontaneous mutagenesis was measured at the transgenic gpt locus. It was therefore important to rule
out effects on mutagenesis which might be unique for this transgene. For this purpose, V79 cells were grown in medium containing the same range of Zn(II) concentrations and mutations were measured at the endogenous hprt locus (Fig. 5C). V79 cells have a lower spontaneous mutation frequency at the hprt locus compared with G12 cells at the gpt locus (17). However, V79 cells showed the same response to Zn(II) as did the G12 cells. At 12 μM Zn(II), a 3-fold increase in the mutant fraction was observed. At higher concentrations, the mutant fraction decreased considerably. Northern analysis revealed a very gradual increase in MT mRNA level when V79 cells were grown in increasing concentrations of Zn(II) with the highest level at 47 μM, where there was no detectable mutagenesis (Fig. 5D).

DISCUSSION

The results of this study demonstrate that the level of MT expression in cells is inversely related to their spontaneous mutability, and that MT is protective against the mutagenicity of zinc. Spontaneous mutagenesis is thought to be the result of various endogenous processes including DNA polymerase errors and oxidation, methylation, deamination, and depurination of DNA (24—26). Our results on induced mutagenesis by MNNG and EMS demonstrate that MT has no effect on the mutagenicity or toxicity of these alkylating agents. Since these agents cause mutations by mispairing during replication of damaged DNA, it is unlikely that MT decreases the level of alkylation-induced DNA damage, or increases the efficiency of DNA repair or the fidelity of DNA replication past lesions. It has been suggested that MT protects cells from free radical damage (27, 28). In support of this concept is our preliminary data (not shown) that MT1—2A cells show increased resistance to the cytotoxic action of menadion, a compound which produces free radicals (29).

It was demonstrated earlier that growth of V79 cells in the presence of increasing concentrations of CdCl2 or ZnCl2 leads to increased expression of MT (8). We show that when G12 and the parental V79 cells are grown in medium containing Zn(II) concentrations that are nontoxic but that induce expression of endogenous MT, significant decreases in spontaneous mutation frequencies were observed. These experiments confirm the data obtained with the MT transfectants and show that either introducing an exogenous MT gene or inducing endogenous MTs results in a reduction of spontaneous mutagenesis. There has been increased attention recently to causes of spontaneous mutations and their consequences. Some recent reviews on this subject suggest that spontaneous mutagenesis plays an important role in baseline incidence of carcinogenesis (25, 26, 30). There is evidence that normal cellular metabolism can be a major source of DNA damage leading to spontaneous mutagenesis and, by extension, to spontaneous carcinogenesis (30). Since there is individual variability in terms of protection against endogenous DNA damage, this might be reflected in variability in the risk of tumor formation (26). It is important to note that many sources of spontaneous mutagenesis are unavoidable. The fact that it is possible to modulate the level of spontaneous mutagenesis by modulating the MT levels argues for a crucial role for MT in protecting cells from some types of mutagenesis.

Another striking result of this study is the mutagenicity of Zn(II) at some relatively low concentrations. Zinc has long been considered to be one of the toxic metals in the environment. When the mutagenic effects of Zn(II) were reviewed in 1986, it was concluded that zinc was not mutagenic (31). Although workers in the zinc industry had been shown to exhibit increased chromosome aberrations in their circulating lymphocytes (32), these effects were attributed to their exposure to other clastogens. A 3-h exposure to Zn(II) did not induce thymidine kinase mutants in mouse lymphoma cells (33). Yet there are some reports pointing to a genotoxic action of Zn(II). Zn(II) was found to be mutagenic in an evaluation of the Salmonella typhimurium mutagenesis assay by the United State Environmental Protection Agency (34). Administration (i.p.) of ZnCl2 to Swiss albino mice induced a significant dose-dependent increase in chromosomal aberrations in bone marrow cells at all concentrations tested and caused sperm head abnormalities (35). Treatment of human fibroblasts with high concentrations of Zn(II) caused the appearance of DNA strand breaks which were rapidly repaired (36). Much of these data have been ignored because of the generally held view that exposure to zinc presents no risk.

Zinc is involved in many endogenous cellular processes. The function of numerous proteins, especially those involved in DNA binding, depends on the presence of zinc (3). MT within cells appears to be mainly cytoplasmic, but immunohistochemical localization studies have shown that the protein is also present within hepatic and renal nuclei (37). Cadmium-resistant fibroblast cells have been shown to stain intensely for MT in both the nuclear and cytoplasmic compartments (38). It has been suggested for many years that MT may function in the regulation of zinc metabolism, i.e., its storage, transport, and distribution. Inhibition of the binding of transcription factors TFIIIA and Sp1 to DNA could be accomplished by sequestering of their zinc by MT (39). However, such functions have not been demonstrated in vivo.

The mechanism of mutagenesis by Zn(II) is unknown. In contrast to the very high concentrations (>500 μM) of Zn(II) used to induce strand breaks in human fibroblasts (36), the mutagenic effects observed in this study occurred at very low concentrations of zinc. Preliminary data from this laboratory suggests that there is a significant increase in the level of oxidative species detected in G12 cells in the same Zn(II) concentrations which elicit increased spontaneous mutagenesis. At higher concentrations of Zn(II), when MT is induced, the level of oxidants decreases. These data suggest that zinc, unopposed by MT, causes oxidative damage to DNA by some unknown mechanism.

In light of these results, the biological function of MT must be reexamined. Since its discovery, it has been thought that the main function of MT is detoxification of heavy metals (1). Our data demonstrate a possible role of MT in modulating spontaneous mutagenesis and point to a role for Zn(II) in contributing to spontaneous mutagenesis. In the absence of sufficient levels of MT, zinc can become mutagenic. This might be one of the main causes of spontaneous mutations, and could explain why MT is so well conserved. We suggest that MT evolved to play a protective role against mutagenesis by zinc. This hypothesis does not exclude another role for MT in delivering zinc to enzymes and other proteins. However, it may be important to maintain a normal zinc balance in the nucleus, not only to provide zinc when it is needed but also to protect DNA from its genotoxicity, since a sudden increase in the intracellular zinc concentration could lead to DNA damage. The nature of the mutations which MT prevents is not known. Polymerase chain reaction analysis of spontaneous G12 variants selected in 6-thioguanine revealed that deletion had occurred with a frequency of approximately 25% (23). While it is possible for the gpt transgene in G12 to be inactivated by changes in methylation, such inactivation accounts for only around 10% of the spontaneously arising 6-thioguanine-resistant variants.5 Since the frequency of spontaneous gpt variants was decreased 80—90% in cells expressing high levels of MT, a large fraction (if not all) of the reduction must be accounted for by a reduction in mutagenesis. At present, efforts are under way to determine the types of DNA

5 Y. W. Lee et al., manuscript in preparation.
damage and to sequence the spontaneous mutants arising under the conditions studied here. There is a significant amount of individual variability in the mutant fraction at the hprr locus in human T lymphocytes (40). It is reasonable to assume that variability in spontaneous mutagenesis accounts for at least part of the variability in the mutant fraction. There is also significant intrasubject variability in both basal and induced levels of MT mRNA in peripheral human lymphocytes (41). We hypothesize that variability in MT expression might play a role in the variability of spontaneous mutagenesis in humans and possibly of spontaneous carcinogenicity as well. Human diets are also variable with regard to zinc content (42) and many people take dietary supplements of zinc. In light of the role of zinc in spontaneous mutagenesis, the safety of zinc supplements should be reconsidered.

ACKNOWLEDGMENTS

We thank Ms. Eleanor Cordisco for her expert help in document preparation.

REFERENCES

A Role for Metallothionein and Zinc in Spontaneous Mutagenesis

Ekaterina I. Goncharova and Toby G. Rossman


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/54/20/5318

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.