Overexpression of Metallothionein in Chinese Hamster Ovary Cells and Its Effect on Nitrogen Mustard-induced Cytotoxicity: Role of Gene-specific Damage and Repair

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ABSTRACT

Overexpression of metallothionein in mammalian cells has been associated with protection from cytotoxic chemicals and acquired resistance of tumors to cytotoxic drugs. The mechanism of this effect, however, remains unclear. We have explored whether cytotoxicity of the bifunctional alkylating agent nitrogen mustard was correlated with the extent of DNA damage formation and repair in the metallothionein gene regions in Chinese hamster ovary cells. The DNA damage and repair were examined in metallothionein-overexpressing, cadmium-resistant Chinese hamster ovary cells, Cd200T1, with or without zinc-induced transcriptional activation, and in the parental CHO-met− cell line. The zinc-induced Cd200T1 cells tolerated significantly higher doses of nitrogen mustard than did the uninduced Cd200T1 variant. The parental CHO-met− cells, which did not have any detectable metallothionein expression, were even more resistant to nitrogen mustard than the zinc-induced Cd variants. Nitrogen mustard-induced N-alkylpurines were formed with a higher frequency in inactive genomic regions than in the active genes. The removal of N-alkylpurines was similar in the active MT I gene region in Cd200T1 and the silent MT I gene region in the parental cells, and the expression of these genes was determined by Northern assay. The MT II gene-containing region was repaired less efficiently than the MT I gene, independently of zinc induction. Further, preferential repair of nitrogen mustard-induced N-alkylpurines were detected in a single copy of the essential active dihydrofolate reductase gene as compared to a downstream noncoding region. This preferential repair was unaffected by the presence of zinc. Neither damage formation nor repair kinetics in the MT gene regions seemed to parallel the observed spectrum of sensitivity to HN2.

INTRODUCTION

MT4 are cysteine-rich proteins that constitute the major fraction of the intracellular protein thiols. The abundance of cysteine predisposes MT toward binding of Hg metals and functioning extensively in the homeostasis of essential metals such as zinc and copper and heavy metal detoxification (for review see Ref. 1). In addition, MT are inducible and it has been generally assumed that the abundant nuclophilic thiol-rich groups in MT can react with many electrophilic toxins, participate in controlling the intracellular redox potential, and act as scavengers of oxygen radicals generated during the metabolism of xenobiotics (1). This hypothesis is indirectly supported by the observation that the level of MT is increased in response to various forms of cytotoxic stress induced by UV light (2), ionizing radiation (3), anticancer agents (4), or carcinogens (5).

Previous experiments have shown that transfection of the human MT II-A gene into CHO-K1 cells produces a significant increase in resistance to the monofunctional alkylating agents N-methyl-N'-nitro-N-nitrosoguanidine and N-methyl-N-nitrosourea, but not to methyl methane-sulfonate or N-hydroxyethyl-N-chloroethylnitrosourea (6). In a similar way overexpression of MT conferred resistance to various alkylating anticancer agents (7, 8). These results did not, however, clarify the direct role of MT, if any, in this form of drug resistance.

Nitrogen mustards are a group of highly electrophilic anticancer agents which react with the native DNA in a sequence-specific manner. These alkylating agents react predominantly with guanines by alkylation of their N-7 positions or with GC-rich regions to form DNA interstrand cross-links (9, 10). Their biological effects may depend upon preferential reaction at certain genomic regions. It appears that the location of DNA adducts greatly influences the rate with which they are repaired. Thus, the excision repair of some DNA lesions in mammalian chromatin is heterogeneous. Preferential repair of UV light-induced cyclobutane pyrimidine dimers and cisplatin-induced DNA cross-links from active transcribing regions has recently been shown to be a determinant of cytotoxicity of DNA damaging agents (11, 12). Also, preferential repair of HN2-induced N-alkylpurines have been shown to resemble that seen for the removal of UV light-induced DNA adducts (13). Whether this preferential repair of HN2-induced alkylation in active genes is a general determinant of cytotoxicity remains to be clarified.

Crawford et al. (14) established a number of cadmium-resistant CHO cell lines including the variant Cd′200T1 which was found to carry an amplification of the MT genes and to express increased amounts of metallothionein. After induction with subtoxic concentrations of ZnCl2, the level of metallothionein increased further (15). In agreement with previous studies (6–8) we here report that the zinc-induced Cd′200T1 variant was about 2-fold more resistant to HN2-induced cytotoxicity than the uninduced Cd′200T1 cells. The parental CHO-met− cells, however, which did not have any detectable MT expression, were severalfold more resistant than both the uninduced and induced Cd′ variant. In the present study we have used a recently developed methodology to analyze whether the observed differential sensitivity to HN2 was coordinately related to the extent of HN2-induced N-alkylpurine production and removal in specific genomic regions containing the MT I and MT II genes in the MT overexpressing Cd′200T1 CHO variant and in the parental CHO-met− cell line.

MATERIALS AND METHODS

Isotopes, Drug, Enzymes, and Hybridization Probes. [methyl-3H]-Thymidine (>80 Ci/mmol) and [α-32P]dCTP (3000 Ci/mmol) were purchased from Du Pont-New England Nuclear. Bis(2-chloroethyl)
methylamine (nitrogen mustard) was obtained through the Developmental Therapeutics Program, Division of Cancer Treatment, National Cancer Institute, and was dissolved immediately prior to use in 0.1 M HCl. Restriction endonucleases (BamHI, KpnI, and HindIII) and pBR322 were purchased from Boehringer Mannheim and International Biotechnologies, Inc., New Haven, CT, respectively. Cd200T1 cells, the parental cell line CHO-met- and complementary DNA probes for MT I and MT II were generously provided by Dr. C. E. Hildebrand, Los Alamos National Laboratory, University of California, New Mexico. The probes have been described by Griffith et al. (16). All probes for the DHFR gene (pMB5) and the downstream, noncoding region (cs-14DO) have been described (17). The pMB5 probe was used to detect the CHO DHFR 14-kilobase KpnI fragment, and a subclone of cs-14 (cs-14DO) was used to detect the downstream, noncoding 14-kilobase KpnI fragment of the DHFR gene. The cs-14DO probe was generated by selecting for fragments which contained a minimum of repetitive sequences.

Cell Culture. Cd200T1 cells carrying an amplification of the MT genes were grown in minimal essential medium (Gibco Laboratories) supplemented with 10% fetal calf serum, nonessential amino acids, penicillin-streptomycin, and 10 μM ZnCl2 in humidified 5% CO2-95% air at 37°C (14). Cd200T1 cells with induced MT genes were prepared by preincubation in the presence of 100 μM ZnCl2 for 2 h and subsequently during survival and repair experiments (18). The parental CHO-met- cells were grown in a similar medium without ZnCl2. Cell cultures for all experiments in this study were in exponential growth phase at the time of DNA damage.

Colony-forming Ability. Single cell suspensions were obtained by gentle trypsinization of adherent cultures, and cells were plated at 5 × 103 B-100-mm dish. After 48 h of incubation at 37°C, cells were washed once with phosphate-buffered saline at room temperature and reincubated in minimal essential medium supplemented with 1% fetal calf serum; thereafter, HN2 was added at different concentrations, and the cells were incubated at 37°C for 30 min. Following drug exposure, cells were washed twice with phosphate-buffered saline at room temperature and gently trypsinized. The cells were then resuspended in minimal essential medium with 10% fetal calf serum at 40°C. These cells were plated at 5 × 103/100-mm dish. After 48 h of incubation, the colonies were stained with methylene blue, and the number of colonies containing more than 50 cells was counted. Untreated controls from each culture were run in parallel; plating efficiency for the untreated cells was >80%.

Drug Treatment, DNA Isolation, and Southern Blot Analysis. Cellular DNA was prelabeled by allowing cells to grow for 64 h at 37°C in [3H]thymidine (0.3 μCi/ml) and 10 μM "cold" thymidine. Following this procedure, the cells were subcultured and incubated in label-free medium for 24 h. The steps involved in the measurement of DNA damage and repair in specific genomic regions have been described in detail (13). Briefly, the prelabeled cells were treated with HN2 at 37°C for 30 min in fresh medium supplemented with 1% fetal calf serum. At the end of drug exposure, cells were washed with phosphate-buffered saline and either lysed (in 5 ml of 4 M guanidium isothiocyanate 25 mM sodium citrate pH 7.0-0.5% sodium-N-laurylsarcosine-0.1 M β-mercaptoethanol) immediately or after posttreatment incubation for 1, 2, 4, 8, 12, or 24 h. Total cellular RNA from cultured cells was extracted by the single step method of Chomczynski and Sacchi (20). RNA was resuspended in diethyl pyrocarbonate-treated 10 mM Tris pH 7.4-1 mM EDTA and quantitated by absorbance measurements at 260 nm. RNA from control and drug-treated cells was denatured by heating at 60°C in 50% formamide, 6% formaldehyde, and 1× electrophoresis buffer (20 mM morpholinopropanesulfonic acid, 5 mM sodium acetate, and 1 mM EDTA, pH 7.0) for 5 min. Identical amounts of denatured RNA (10 μg/sample) were size-fractionated through 1.2% (w/v) agarose gels containing 2.0 mM formaldehyde and 1× electrophoresis buffer. The gels were run at 50 V for about 5 h, and the RNA was transferred to GeneScreen Plus membranes (DuPont) by capillary transfer in 20× SSC overnight. The membranes were washed in 2× SSC and baked in a vacuum oven at 80°C for 2 h.

Prehybridization of the membranes was carried out in 50% formamide, 5× SSC. 10 mM Tris (pH 7.5), 0.2% sodium dodecyl sulfate, 1× Denhardt’s solution without bovine serum albumin (0.02% Ficoll 400 and 0.02% polyvinylpyrrolidone), and 125 μg/ml denatured salmon sperm DNA at 42°C for 6-24 h. Hybridization was carried out in the same prehybridization solution, at 42°C for 18-24 h, to which denatured 32P-labeled probe had been added. Following hybridization, the membranes were first washed in 2× SSC-0.1% sodium dodecyl sulfate-2.5 mM EDTA and then in 1× SSC-0.05% sodium dodecyl sulfate-1.25 mM EDTA at room temperature for 30 min, respectively. The membranes were exposed to Kodak X-Omat AR film (Eastman Kodak Co.), and band intensities were quantified using a Shimadzu dual-wavelength thin layer chromatoscaner model CS-930.

RESULTS

Cell Survival after HN2 Exposure. Survival of the Cd200T1 cells after exposure to various doses of HN2 measured as their ability to form colonies was determined after cultivation in the presence or absence of ZnCl2. Survival of the parental CHO-met- cells to HN2 was measured in the absence of ZnCl2. Colony survival curves were obtained for 30-min exposures to HN2 and demonstrated a dose-dependent and exponential loss of colony-forming ability (Fig. 1). The zinc-induced Cd200T1 cells [MT overexpressors (14)] were markedly more resistant to HN2 than the uninduced Cd200T1 cells; the 50% lethal concentration value was calculated to 0.35, 0.63, and 3.14 μM for HN2 in the uninduced Cd200T1 cells, the zinc-induced Cd200T1 cells, and the CHO-met- cells, respectively [Table 1].

Dose-dependent Alkylation in Fragments Containing the MT I and the MT II Genes. The extent of HN2-induced N-alkylpurines in the MT I and in the MT II gene loci were analyzed. Isolated DNA from parental cells and from the Cd200T1 variant treated with HN2 was restricted with BamHI and analyzed after neutral depurination followed by alkaline hydrolysis as described above. The production of AP sites was linear with HN2 dose in both cell lines (Fig. 2). The initial lesion frequency in the MT I and MT II genes of the MT-overexpressing Cd200T1 variant was significantly less than that in the more resistant parental cell line [P < 0.005, Wilcoxon test (Table 1)]. Also, the initial adduct frequency in MT I and MT II genes were less in the uninduced Cd200T1

5 D. Okumoto, unpublished information.
cells than that in the zinc-induced Cd²⁺ variant (Table 1). Resistance to HN2 of the MT-induced cells and the parental CHO-met− cells was therefore not associated with a reduction in the initial frequency of N-alkylpurines in the MT I and MT II genes.

In order to determine whether the intracellular availability of HN2 differed between the Cd²⁺ variant and parental cells, analysis of HN2-induced initial lesion frequency were carried out in the single copy DHFR gene and the 3'-flanking nontranscribing region; these genomic regions should not be affected by the transcriptional status of metallothionein. The initial adduct frequency of HN2 in both the 5'-half of the DHFR gene and 3'-flanking noncoding region were similar in zinc-induced Cd²⁺200T1 cells and in the parental CHO-met− cells (Table 1). However, the initial adduct frequencies in the most sensitive and uninduced Cd²⁺200T1 cells were less than in both the induced Cd²⁺200T1 cells and parental cells. HN2-induced alkali-labile lesion frequency in the 5'-half of the DHFR gene, however, was consistently less than in the 3'-flanking region in both the Cd²⁺ variant and the parental cell line (Table 1).

Repair of N-Alkylpurines in Fragments Containing the MT I and MT II Genes, the DHFR Gene and in Its 3'-Flanking Nontranscribing Region. Heterogeneous DNA repair has been shown to be a determinant of cytotoxicity of DNA-damaging agents (11, 12). We therefore proceeded to examine the removal of HN2-induced N-alkylpurines from the regions of interest in the Cd²⁺200T1 and CHO-met− cells. After treatments of cells with 150 µM HN2, repair incubation for 0, 4, 8, and 24 h, isolation, and purification of parental DNA as described, the repair kinetics was determined in the fragments containing the MT I and MT II gene regions, the 5'-half of the DHFR gene and in the 3'-flanking region of the DHFR gene. In the 6-kilobase BamHI fragment with the MT I gene, we found very efficient DNA repair within 24 h after HN2 exposure of the Cd²⁺200T1 cells (Figs. 3 and 4; Table 2). This efficient repair was not affected by the zinc induction of the gene. Furthermore, the repair efficiency in the MT I gene was similar in the Cd²⁺ variant and in the parental CHO-met− cell line. The MT II-containing fragment was repaired less efficiently after 24 h than the MT I region (Table 2), and its repair was not affected by the induction.

In order to determine whether addition of ZnCl₂ changed the general DNA repair characteristics of the cells, repair measurements were carried out in a gene where expression should not be affected by ZnCl₂. We measured the repair efficiency of HN2-induced N-alkylpurines in the essential, constitutively active single copy DHFR gene, and it was similar in Cd²⁺200T1 in the absence and presence of ZnCl₂ and in the parental CHO cell line (Table 2). The removal of HN2 adducts in the nontranscribing 3'-flanking region of the DHFR gene, however, was considerably less efficient than in the DHFR gene (Figs. 3 and 4; Table 2).

Metallothionein Transcription following HN2 Exposure. We have reported previously that a number of DNA lesions are repaired more rapidly from actively transcribing genes than from nontranscribed regions or from the genome overall in cultured cells (12, 13, 21–23). In order to determine whether repair efficiency of N-alkylpurines in the MT gene-containing

### Table 1
Nitrogen mustard-induced cytotoxicity and alkali-labile lesion frequency in different regions of the CHO genome in uninduced Cd²⁺200T1, ZnCl₂-induced Cd²⁺200T1, and CHO-met− cell lines

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Cytotoxicity a (IC₅₀, µM)</th>
<th>ZnCl₂</th>
<th>MT I (6 kilobases)</th>
<th>MT II (7 kilobases)</th>
<th>DHFR (14 kilobases)</th>
<th>3'-flanking (14 kilobases)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cd²⁺200T1</td>
<td>0.35 ± 0.06</td>
<td>−</td>
<td>1.22</td>
<td>1.54</td>
<td>1.11</td>
<td>1.21</td>
</tr>
<tr>
<td>CHO-met−</td>
<td>0.63 ± 0.04</td>
<td>+</td>
<td>1.59 ± 0.14</td>
<td>1.76</td>
<td>1.39 ± 0.21</td>
<td>1.75 ± 0.14</td>
</tr>
<tr>
<td></td>
<td>3.14 ± 0.20</td>
<td></td>
<td>3.66 ± 0.25</td>
<td>1.32 ± 0.12</td>
<td>1.68 ± 0.15</td>
<td></td>
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</table>

a Values represent the mean ± SE of three to six independent experiments, in which duplicate samples were measured in each. Only the mean value was shown when less than three independent experiments were done.
b IC₅₀, 50% inhibitory concentration.
I and digested genomic fragments containing the repair-incubated are 0, 4, 8, and 24 h. The and ZnCl₂-induced Cdr200Tl cells probed for (a) with 32p-labeled pMB5 and modified cs-14DO, respectively. The panels shown are autoradiograms of the Southern blots of genomic DNA from uninduced Cdr200T1 (nondepurinated) control (- cleavage) and heated (depurinated) control (+ cleavage) are shown. DNA in duplicate lanes from cells treated with 150 µM HN2 and MT II with 150 /µM HN2 for 30 min at 37°C in medium containing 1% serum were digested with KpnI-digested genomic fragments containing the MT I (6-kilobase) and MT II (7-kilobase) gene regions were probed with 32P-labeled complementary DNA probes for MT I and MT II. KpnI-digested genomic fragments containing the 5'-half of the DHFR gene (14 kilobases) and the 3'-flanking noncoding region (14 kilobases) were probed with 32P-labeled pMB5 and modified cs-14DO, respectively. The panels shown are autoradiograms of the Southern blots of genomic DNA from nonheated (nondepurinated) control (- cleavage) and heated (depurinated) control (+ cleavage) are shown. DNA in duplicate lanes from cells treated with 150 µM HN2 and repair-incubated are 0, 4, 8, and 24 h. The MT II-containing region is partly homologous to the MT I-containing region.

No expression of MT I RNA was observed in the parental CHO-met- cells (Fig. 5, top). β-Actin was expressed (at similar levels) in both Cd²⁺200T1 cells and CHO-met- cells. The exposure of cells to HN2 in this study appeared to have almost no effect on β-actin expression over the first 4–8 h. Then the β-actin transcripts gradually fell below the basal levels observed in the control cultures 24 h after drug exposure to about 47 and 45% in Cd²⁺200T1 cells and CHO-met- cells, respectively (Fig. 5).

**DISCUSSION**

Recently, experiments have suggested that increased MT content can render tumor cells resistant to a subset of clinically important anticancer agents (7, 8) and that MT expression is implicated in the generation of resistance to certain monofunctional alkylating agents (6). It was suggested that MT participates as a cofactor or regulatory element in the repair or tolerance of toxic alkylation lesions.

We have explored whether differential cytotoxicity of the bifunctional alkylating agent HN2 was coordinately related to the level of DNA adducts and repair in specific genomic regions containing the MT I and MT II genes. This was done in a MT-expressing Cd²⁺200T1 CHO variant in the absence and presence of the inducing agent ZnCl₂, and in the parental CHO-met- cell line. We find that zinc-induced Cd²⁺200T1 cells were about 2-fold more resistant to HN2 cytotoxicity than the uninduced Cd²⁺ variant. Yet, the parental CHO-met- cells, which did not have any detectable MT expression, were markedly more resistant to HN2 than both the uninduced and the induced Cd²⁺200T1 cells. We find that HN2-induced N-alkylpurines are (a) heterogeneously distributed among the MT gene regions with a higher initial lesion frequency per kilobase DNA fragments was associated with transcriptional activity we examined the effect of HN2 on steady state MT I mRNA during repair incubation.

Fig. 5 shows the effect of HN2 on MT I steady state transcripts in the zinc-induced Cd²⁺200T1 cells and the parental CHO-met- cells, as measured by Northern blot analysis. Treatment with the same HN2 concentration as in the repair experiments (150 µM) produced an immediate decrease of the MT I transcripts to about 65% of the initial level in the untreated control Cd²⁺200T1 cells. Within 2 h following drug removal the MT I transcripts returned almost to control levels and then returned to the initial level (Fig. 5).

Fig. 3. Autoradiograms for analysis of repair of HN2-induced N-alkylpurines in specific genomic regions in CHO cells. DNA from cells that had been treated with 150 µM HN2 for 30 min at 37°C in medium containing 1% serum were digested with BamHI or KpnI and prepared as described in “Materials and Methods.” BamHI digested genomic fragments containing the MT I (6-kilobase) and MT II (7-kilobase) gene regions were probed with 32P-labeled DNA probes for MT I and MT II. KpnI-digested genomic fragments containing the 5'-half of the DHFR gene (14 kilobases) and the 3'-flanking noncoding region (14 kilobases) were probed with 32P-labeled DNA probes for MT I and MT II. KpnI-digested genomic fragments containing the 5'-half of the DHFR gene (14 kilobases) and the 3'-flanking noncoding region (14 kilobases) were probed with 32P-labeled DNA probes for MT I and MT II. The panels shown are autoradiograms of the Southern blots of genomic DNA from uninduced Cdr200T1 and ZnCl₂-induced Cdr200T1 cells probed for (a) MT I, (b) MT II, (c) 5'-half of the DHFR gene, and (d) noncoding region. DNA in duplicate lanes from nonheated (nondepurinated) control (- cleavage) and heated (depurinated) control (+ cleavage) are shown. DNA in duplicate lanes from cells treated with 150 µM HN2 and repair-incubated are 0, 4, 8, and 24 h. The MT II-containing region is partly homologous to the MT I-containing region.

Fig. 4. Autoradiograms for analysis of repair of HN2-induced N-alkylpurines in specific genomic regions in CHO cells. DNA from cells that had been treated with 150 µM HN2 for 30 min at 37°C in medium containing 1% serum were digested with BamHI or KpnI and prepared as described in “Materials and Methods.” BamHI digested genomic fragments containing the MT I (6-kilobase) and MT II (7-kilobase) gene regions were probed with 32P-labeled DNA probes for MT I. KpnI-digested genomic fragments containing the 5'-half of the DHFR gene (14 kilobases) and the 3'-flanking noncoding region (14 kilobases) were probed with 32P-labeled DNA probes for MT I. The panels shown are autoradiograms of the Southern blots of genomic DNA from uninduced Cdr200T1 and ZnCl₂-induced Cdr200T1 cells probed for (a) MT I, (b) MT II, (c) 5'-half of the DHFR gene, and (d) noncoding region. DNA in duplicate lanes from nonheated (nondepurinated) control (- cleavage) and heated (depurinated) control (+ cleavage) are shown. DNA in duplicate lanes from cells treated with 150 µM HN2 and repair-incubated are 0, 4, 8, and 24 h. The MT II-containing region is partly homologous to the MT I-containing region.

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We have explored whether differential cytotoxicity of the bifunctional alkylating agent HN2 was coordinately related to the level of DNA adducts and repair in specific genomic regions containing the MT I and MT II genes. This was done in a MT-expressing Cd²⁺200T1 CHO variant in the absence and presence of the inducing agent ZnCl₂, and in the parental CHO-met- cell line. We find that zinc-induced Cd²⁺200T1 cells were about 2-fold more resistant to HN2 cytotoxicity than the uninduced Cd²⁺ variant. Yet, the parental CHO-met- cells, which did not have any detectable MT expression, were markedly more resistant to HN2 than both the uninduced and the induced Cd²⁺200T1 cells. We find that HN2-induced N-alkylpurines are (a) heterogeneously distributed among the MT gene regions with a higher initial lesion frequency per kilobase DNA.
Removal of HN2-induced alkali-labile lesions was measured in DNA extracted from uninduced Cd°200T1, ZnCl₂-induced Cd°200T1, and CHO-met− cells treated with 150 μM HN2 for 30 min, repair-incubated for 0, 4, 8, and 24 h, restricted with BamHI or KpnI, and Southern blotted prior to probing membranes with 32P-random primed-labeled complementary DNA probes for the MT I- and MT II-containing regions (BamHI digests), and pMB5 for the 5'-half of the DHFR gene and modified cs-14 for the 3'-flanking noncoding region (KpnI digests). Data were derived from densitometry of autoradiograms as described in “Materials and Methods.”

<table>
<thead>
<tr>
<th>Cell line</th>
<th>MT I (6 kilobases)</th>
<th>MT II (7 kilobases)</th>
<th>DHFR (14 kilobases)</th>
<th>3'-Flanking (14 kilobases)</th>
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<tr>
<td>HN2 (150 μM)</td>
<td>Repair (h)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cd°200T1</td>
<td>+ + + + + + + +</td>
<td>+ + + + + + + +</td>
<td>+ + + + + + + +</td>
<td>+ + + + + + + +</td>
</tr>
<tr>
<td>Cho-met−</td>
<td>+ + + + + + + +</td>
<td>+ + + + + + + +</td>
<td>+ + + + + + + +</td>
<td>+ + + + + + + +</td>
</tr>
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% of removal of HN2-induced lesions at following region/gene

<table>
<thead>
<tr>
<th>Cell line</th>
<th>MT I (6 kilobases)</th>
<th>MT II (7 kilobases)</th>
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<tr>
<td>Cd°200T1</td>
<td>47.8 ± 3.0</td>
<td>45.8 ± 2.0</td>
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<td>50.3 ± 2.0</td>
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<tr>
<td>Cho-met−</td>
<td>37.0 ± 2.0</td>
<td>27.0 ± 2.0</td>
<td>28.7 ± 2.0</td>
<td>19.5 ± 2.0</td>
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Fig. 5. Northern blot analysis of the expression of MT I mRNA and β-actin in ZnCl₂-induced Cd°200T1 and parental CHO-met− cells. A, MT I expression and β-actin expression in Cd°200T1 cells. B, MT I expression and β-actin expression in parental CHO-met− cells. C, MT I expression and β-actin expression in Cd°200T1 and parental CHO-met− cells at specific times after drug treatment. Data were derived from densitometry analysis of Northern blots and are presented as mean values from duplicate determinations made within one experiment.

in silent nontranscribing MT I genes and (b) preferentially repaired in the 5'-half of the essential, constitutively active DHFR gene when compared to a noncoding downstream 3'-flanking region to the gene; (c) in contrast to the situation for the DHFR gene, the removal of HN2-induced alkylation was similar in the different MT gene-containing fragments and unaffected by the transcriptional activity of these genes.

The observation that there is increased resistance of MT-overexpressing cells towards the anticancer agents HN2 (our data), cis-diaminedichloroplatinum, melphalan, and chlorambucil (7, 8) does not support the idea that there is a selective protective cellular response to agents with a high ratio of O6/N7 alkylation of guanines (6, 24). In addition to the monoalkylation of guanine N7 positions, nitrogen mustards also produce guanine intrastrand and interstrand cross-links as well as protein-DNA crosslinks (10, 25–28). The cross-links, which are the likely mediators of most of the cytotoxicity, are all believed to involve predominant alkylation of N7-guanine. Also, these cytotoxic anticancer agents have been reported to form low levels of O6-alkylation (29).

In the Cd° variant, the level of HN2-induced alkylation adducts were independent of MT gene induction. Also, the homogeneous alkylation pattern in both the DHFR gene and in the 3'-flanking region of the cell lines studied ruled out differences in transport of HN2. Induction of more alkali-labile sites was observed in the inactive MT I-containing region than in the transcriptional active fragment of the same gene in Cd°200T1. Also, consistent with our recent findings (13), heterogeneity in damage formation was observed between the active DHFR gene and the noncoding region 3' to the gene in both the Cd° variant and the parental cells (Table 1). This finding is in contrast to the reports on higher initial lesion frequency by N-nitrosodimethylamine (30) and psoralen photoadducts (31) in more open regions of chromatin, since noncoding regions are likely to be less open in their chromatin structural configuration than active genes. Also, our findings with HN2 are in opposition to the previous reports of homogeneous distribution of N-methylpurines within active transcribing and inactive genomic regions after exposure of cultured rodent cells to dimethyl sulfate (13, 32), N-methyl-N-nitrosourea (23) and human fibroblasts to N-methyl-N'-nitro-N-nitrosoguanidine (33). In a recent report, two expressed genes contained slightly more alkali-labile sites than did an inactive gene after exposure of cultured human T-lymphocytes to methyl methanesulfonate (34). This suggests that there are differences in the distribution of monofunctional and bifunctional alkylation agents and further that there may be interorgan and/or interspecies differences in alkylation bias.

Table 2 Repair of nitrogen mustard-induced alkali-labile lesions in different regions of the CHO genome in uninduced Cd°200T1, ZnCl₂-induced Cd°200T1, and CHO-met− cell lines

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</tr>
<tr>
<td>Cho-met−</td>
<td>+ + + + + + + +</td>
<td>+ + + + + + + +</td>
<td>+ + + + + + + +</td>
<td>+ + + + + + + +</td>
</tr>
</tbody>
</table>

% of removal of HN2-induced lesions at following region/gene

<table>
<thead>
<tr>
<th>Cell line</th>
<th>MT I (6 kilobases)</th>
<th>MT II (7 kilobases)</th>
<th>DHFR (14 kilobases)</th>
<th>3'-Flanking (14 kilobases)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cd°200T1</td>
<td>47.8 ± 3.0</td>
<td>45.8 ± 2.0</td>
<td>65.0 ± 2.0</td>
<td>50.3 ± 2.0</td>
</tr>
<tr>
<td>Cho-met−</td>
<td>37.0 ± 2.0</td>
<td>27.0 ± 2.0</td>
<td>28.7 ± 2.0</td>
<td>19.5 ± 2.0</td>
</tr>
</tbody>
</table>

Fig. 5. Northern blot analysis of the expression of MT I mRNA and β-actin in ZnCl₂-induced Cd°200T1 and parental CHO-met− cells. A, MT I expression and β-actin expression in Cd°200T1 cells. B, MT I expression and β-actin expression in parental CHO-met− cells. C, MT I expression and β-actin expression in Cd°200T1 and parental CHO-met− cells at specific times after drug treatment. Data were derived from densitometry analysis of Northern blots and are presented as mean values from duplicate determinations made within one experiment.

in silent nontranscribing MT I genes and (b) preferentially repaired in the 5'-half of the essential, constitutively active DHFR gene when compared to a noncoding downstream 3'-flanking region to the gene; (c) in contrast to the situation for the DHFR gene, the removal of HN2-induced alkylation was similar in the different MT gene-containing fragments and unaffected by the transcriptional activity of these genes.

The observation that there is increased resistance of MT-overexpressing cells towards the anticancer agents HN2 (our data), cis-diaminedichloroplatinum, melphalan, and chlorambucil (7, 8) does not support the idea that there is a selective protective cellular response to agents with a high ratio of O6/N7 alkylation of guanines (6, 24). In addition to the monoalkylation of guanine N7 positions, nitrogen mustards also produce guanine intrastrand and interstrand cross-links as well as protein-DNA crosslinks (10, 25–28). The cross-links, which are the likely mediators of most of the cytotoxicity, are all believed to involve predominant alkylation of N7-guanine. Also, these cytotoxic anticancer agents have been reported to form low levels of O6-alkylation (29).

In the Cd° variant, the level of HN2-induced alkylation adducts were independent of MT gene induction. Also, the homogeneous alkylation pattern in both the DHFR gene and in the 3'-flanking region of the cell lines studied ruled out differences in transport of HN2. Induction of more alkali-labile sites was observed in the inactive MT I-containing region than in the transcriptional active fragment of the same gene in Cd°200T1. Also, consistent with our recent findings (13), heterogeneity in damage formation was observed between the active DHFR gene and the noncoding region 3' to the gene in both the Cd° variant and the parental cells (Table 1). This finding is in contrast to the reports on higher initial lesion frequency by N-nitrosodimethylamine (30) and psoralen photoadducts (31) in more open regions of chromatin, since noncoding regions are likely to be less open in their chromatin structural configuration than active genes. Also, our findings with HN2 are in opposition to the previous reports of homogeneous distribution of N-methylpurines within active transcribing and inactive genomic regions after exposure of cultured rodent cells to dimethyl sulfate (13, 32), N-methyl-N-nitrosourea (23) and human fibroblasts to N-methyl-N'-nitro-N-nitrosoguanidine (33). In a recent report, two expressed genes contained slightly more alkali-labile sites than did an inactive gene after exposure of cultured human T-lymphocytes to methyl methanesulfonate (34). This suggests that there are differences in the distribution of monofunctional and bifunctional alkylation agents and further that there may be interorgan and/or interspecies differences in alkylation bias.
At present, the mode by which HN2 produces heterogeneous alkylation is not understood. Since the GC content and base composition of the MT genes should be similar in the Cd200T1 and parental cells other aspects of chromatin structure may regulate damage formation. It is noteworthy that in a recent study on DNA damage and repair of individual DNA strands in the CHO DHFR gene there were about 20% more HN2-induced N-alkylpurines formed in the nontranscribed strand (43). We do not ascribe this heterogeneity in HN2-induced lesion frequency to differences in primary sequence of the actual genomic regions.

The sensitivity to HN2 indicates that a yet unknown protection mechanism functions through a postalkylation event. Preferential repair of specific DNA adducts from actively transcribing regions has recently been shown to be a determinant of cytotoxicity of DNA-damaging agents (11, 12). Also, preferential repair of HN2-induced alkylation resembles that seen for the removal of pyrimidine dimers and 6-4 photoproducts from these genomic regions in CHO cells irradiated with UV light (13, 21, 22). Furthermore, increased DNA repair of UV-induced pyrimidine dimers was also seen in the induced MT I gene of Cd200T1 cells, and in the active human metallothionein gene (18, 35). Thus, our observation that the repair of HN2 induced lesions in the MT genes of Cd200T1 cells are unaffected by zinc induction is in contrast with the situation with preferential repair of UV-induced pyrimidine dimers in these cells. Moreover, the repair of MT I in the Cd200T1 cells was of the same order of magnitude as the repair in the quiescent MT I gene in the parental CHO cells. In contrast, we find preferential repair of HN2-induced alkylation in the essential single copy DHFR gene compared to the inactive region, and this is in accordance with our earlier results in amplified hamster cells (13). It is possible that changes in the repair of HN2 with transcriptional induction of the MT genes are too small to be detected here or that there is no universal linkage between DNA repair and transcription. Other recent findings from our laboratory would support the notion that gene-specific DNA repair changes can be independent of transcriptional changes (36, 37).

We do not find any direct alkylation-protective effect of MT in vivo. Crawford et al. (14) established the variant Cd200T1 by repeated exposure of CHO cells to escalating doses of cadmium. Cd200T1 was found to carry an amplification of the MT genes to about 12 copies and to express increased amounts of MT mRNA. Incubation of these cells with, e.g., 100 μM ZnCl2, induced coordinate expressions of MT I and MT II genes with an increase of about 1000-fold in MT I and 100-fold MT II, respectively (15). Furthermore, in Cd200T1, MT II, but not MT I, was expressed constitutively, possibly suggesting a selective advantage for elevated MT II expression in the generation of Cd′ variants (14). Hence, the Cd′ variant should show an increase in resistance when compared to the MT-nonexpressing parental cells. This effect should be independent of the zinc induction. Although a protection of Cd200T1 cells was observed in the presence of zinc induction, the level of MT expression did not seem to parallel the degree of protection. No expression of MT I mRNA was observed in the most resistant cells, the parental cell line (Fig. 5). This was an expected finding, since MT I and MT II genes in CHO cells are in a quiescent state and hypermethylated (2, 14).

The fate of toxic alkylation damage in mammalian cells is apparently subject to several pathways. MT production may not solely account for modulation of cytotoxicity by alkylating agents, or even of cadmium and zinc. Studies have indicated the possible involvement of glutathione metabolism and altered binding of MT to non-MT components as factors in cadmium resistance (38, 39). Moreover, recent studies have suggested an association of overexpressed glutathione S-transferase-encoding genes with differential sensitivity to HN2 (40). Finally, recent studies suggest that HN2-induced DNA damage in mammalian cells may elicit a response which results in inhibition of p34cdc2 kinase activity, a protein complex which regulates the transition of cells from G2 to mitosis and is coupled to DNA synthesis and competent DNA repair (41, 42). A resolution of the mechanisms of alkylation cytotoxicity and the role of non-MT components in damage removal or tolerance will be possible only through a characterization of the genes involved and of cellular processes subsequent to removal of damage.

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REFERENCES


Overexpression of Metallothionein in Chinese Hamster Ovary Cells and Its Effect on Nitrogen Mustard-induced Cytotoxicity: Role of Gene-specific Damage and Repair

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