HeLa Cell Transformants Overproducing Mouse Metallothionein Show in vivo Resistance to cis-Platinum in Nude Mice

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Plasmid pSV2MT-I encoding mouse metallothionein-I (MT-I) designed to be expressed under the control of an SV40 promoter was introduced into human HeLa S3 cells. Several transformants (HeLa/MTH) carrying multi-copies of mouse MT-I cDNA in their genomes were isolated. These transformants produced 4 to 20-fold larger amounts of MT than their parent cells. The MT levels in HeLa/MTH were well correlated with the extent of resistance to cadmium, but not with that to cis-platinum (cis-DDP) in vitro. To study the role of MT in resistance to cis-DDP in vivo, nude mice were inoculated subcutaneously with two independent HeLa/MTH clones. MT levels in these tumors were about 3-fold higher than those in the parental cells. The growth of tumors derived from either HeLa/MTH clone was not inhibited in the presence of 15 µmol/kg of cis-DDP, which completely inhibited the growth of tumors derived from the parental HeLa cells. These data strongly suggest that the elevated level of MT confers resistance to cis-DDP in vivo but not in vitro. Thus, the results of this study indicate that in vitro determinations of the influence of MT on cis-DDP resistance may underestimate its importance in in vivo situations.

Key words: Metallothionein — cis-Platinum — Drug resistance — HeLa cell — Overexpression

Cis-diamminedichloroplatinum (cis-DDP), a coordination complex of platinum, is one of the most effective anti-neoplastic agents, with therapeutic activity against a wide variety of human neoplasms.1—3) However, the clinical usefulness of cis-DDP is often restricted by the development of drug resistance. Various mechanisms of cis-DDP resistance have been proposed,4) which include the decreased drug accumulation,5) increased detoxification by sulfhydryl compounds such as glutathione (GSH)6) and metallothionein (MT),7) and increased repair of DNA damage.8) MT is a cysteine-rich protein of low molecular weight, and its synthesis is induced by a number of heavy metals and many other factors.9) MT is known to have multiple functions, such as protection of cells against heavy metal toxicity,9) inactivation of alkylating agents10,11) and scavenging free radicals.12,13) It has been demonstrated that the toxic effects of cis-DDP14—16) adriamycin (ADR),14,17,18) tumor necrosis factor19) and γ-irradiation20) can be significantly reduced by preinduction of MT synthesis in mice. In mice transplanted with tumor cells, induction of MT synthesis in the tumor by administration of a zinc compound has been found to endow tumors with resistance to cis-DDP.21) Endresen et al.22) also reported that transplanted mouse fibroblast cells with high MT content exhibited resistance to cis-DDP. On the other hand, the results of in vitro experiments with cultured tumor cells suggest that intracellular levels of MT are important in determining cellular responsiveness to cis-DDP.23—25) For example, mouse fibroblast C127 cells and CHO cells which overexpressed MT-IIA were reported to be resistant to cis-DDP as well as to some alkylating agents.26—29) However, Schilder et al.30) observed that C127 cell lines with high MT levels were not resistant to cis-DDP. Koropatnick and Pearson31) also reported that CHO cells expressing functional mouse MT-I were more sensitive to cis-DDP toxicity than the parental cells. Recently, Minamino et al.32) reported that rat hepatoma AH66 cells, which have a high ability to induce MT synthesis were highly resistant to cis-DDP in vivo, but not in vitro. To investigate the role of MT in the resistance of tumor cells to cis-DDP in vitro and in vivo, we have constructed plasmid pSV2MT-I, which is designed to express mouse MT-I cDNA under the control of an SV40 promoter. By...
transfection with this expression vector, we have established several stable transformants (HeLa/MTH) which produce larger amounts of MT than the parental cells in the absence of any MT inducer. First, the sensitivity of the cells to various concentrations of cis-DDP was examined in vitro using an 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Secondly, two clones of HeLa/MTH and control cell lines were transplanted into nude mice and the resulting tumors were examined for their sensitivity to cis-DDP in vivo. The transplanted tumors with high MT levels, derived from both HeLa/MTH cell lines showed resistance to cis-DDP in vivo. Studies described herein indicate that an elevated level of MT in tumor cells confers upon them resistance to cis-DDP in vivo, but not in vitro.

MATERIALS AND METHODS

DNA procedure  Restriction enzymes and other enzymes used in subcloning and modifying DNA were obtained from Takara Shuzo Co. (Kyoto). Standard techniques were used to subclone DNA fragments, to identify recombinant plasmids and to purify DNA fragments for use as probes. DNA primers were prepared by a DNA synthesizer (Applied Biosystems, Foster City, CA).

Construction of pSV2MT-I A cDNA (235 nucleotides) which corresponds to the coding region of mouse MT-I mRNA was synthesized by a method involving the polymerase chain reaction (PCR), using a sense primer, 5′-GGCAATTCAGCTTCAGCCAGCGCACCATACTGCA3′, an antisense primer, 5′-GGCAATTCGGATCGCCTGCAGTTA-3′, and transcribed salmon sperm DNA, followed by two washings in 0.1× SSC and 1% SDS at 50°C. Total RNA of mouse L cells as a template. Segments underlined were added to the terminals of the PCR product and used as adaptors. The cDNA thus synthesized was subcloned into the HindIII-BamHI site of pUC119. The nucleotide sequence was determined using Sequenase sequencing kits (Amersham, Cleveland, OH) to confirm that the sequence was identical to the corresponding region of mouse MT-I cDNA reported by Durnam et al.30 This HindIII-BamHI cDNA fragment was inserted into the HindIII-BglII site of a plasmid vector pSV2-β-globin30 so that the cDNA would be expressed under the control of the SV40 early promoter. This construct was designated pSV2MT-I.

Cells and transfection HeLa S3 cells and HeLa/MTH cell lines have been maintained in Dulbecco’s modified minimum essential medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and kanamycin (60 µg/ml). HeLa S3 cells (1×10⁷ cells) which had been plated in 10-cm plastic dishes one day prior to transfection, were transfected with 20 µg of pSV2MT-I DNA together with pSV2gpt plasmid DNA carrying Escherichia coli xanthine-guanine phosphoribosyltransferase (XGPH) cDNA, by the calcium phosphate co-precipitation method. After 12 h transfection, the cells were rinsed, refed with growth medium and then incubated at 37°C for 24 h. A total of 1×10⁶ cells was dispersed on five 10-cm dishes and transformants were selected in media containing 10% FBS, xanthine sodium salt (250 µg/ml, Sigma, St. Louis, MO), hypoxanthine (15 µg/ml), adenine (25 µg/ml), L-glutamine (150 µg/ml), thymidine (10 µg/ml) and mycophenolic acid (10 µg/ml, Sigma).36 MT-overproducing transformants (HeLa/MTH cell lines) were established by limiting dilution and designated HeLa/MT1, HeLa/MT7, HeLa/MT12, HeLa/MT17, HeLa/MT21, HeLa/MT26 and HeLa/MT27. HeLa/C1 and HeLa/C2 were control cell lines transfected with 10 µg of pUC119 together with 10 µg of pSV2gpt plasmid DNA. Plasmid DNAs which were used for transfection of cells were prepared by the lysozyme-Triton procedure37 followed by at least two cycles of CsCl equilibrium density centrifugation, since E. coli proteins are extremely toxic to animal cells.

Southern blot analysis High-molecular-weight DNAs prepared from 7×10⁷ cells each of HeLa/MTH cells and control HeLa cells were purified from total nucleic acids by treatment with proteinase K (Boehringer Mannheim, Mannheim, Germany) and RNase A (Sigma) followed by phenol-chloroform extraction. DNA was digested with EcoRI, separated by electrophoresis in 0.8% agarose gels and transferred to nitrocellulose filters. The blots were hybridized with 32P-labeled mouse MT-I cDNA fragment as a probe, at 42°C for 15 h in 40% formamide, 3× standard saline citrate (SSC), 0.1% sodium dodecyl sulfate (SDS), 10× Denhardt’s solution, and 50 µg/ml of denatured salmon sperm DNA, followed by two washings in 0.1× SSC and 1% SDS at 50°C for 30 min and exposure to Kodak XAR-5 X-ray film at −80°C overnight. The uniformly 32P-labeled probe was prepared using a random primer DNA labeling kit (Takara). 1× SSC contained 0.15 M NaCl and 0.015 M sodium citrate, and 1× Denhardt’s solution contained 0.02% Ficol, 0.02% poly(vinylpyrrolidone) and 0.02% bovine serum albumin.

Northern blot analysis Total RNA was prepared from 1×10⁷ cells of each cell line using the guanidinium thiocyanate/CsCl method.37 Northern blot analysis of total RNA was performed after electrophoresis in 1% agarose gel containing 2.2 M formamide.38 The concentration of RNA was monitored spectrophotometrically (absorbance at 260 nm) and RNA was analyzed by 1% agarose gel electrophoresis after denaturation of RNA with glyoxal37 to confirm that the samples were free of DNA. Forty micrograms of total RNA per lane was loaded on the gel for electrophoresis. RNA was transferred to a nylon membrane filter (“Photo Gene,” Gibco BRL, Rockville, MD) using a vacuum transfer apparatus (BIOCRAFT, Tokyo), then the blot was hybridized, washed and subjected to autoradiography as described above for Southern blot analysis, except that the hybridization buffer contained...
higher concentrations of SDS (1%) and denatured salmon sperm DNA (250 µg/ml).

The intensities of signals I and II in the autoradiogram shown in Fig. 4 were quantitated by an image analyzer (AMBIS, San Diego, CA).

**Cytotoxicity assay** Sensitivity of various cell lines to cis-DDP in vitro was determined by the MTT tetrazolium colorimetric assay. Cells (1×10⁴ cells per well) were plated in a 96-well plate, incubated overnight, and treated with cis-DDP at various concentrations up to 100 µM. The cells were incubated for 24 h, and each well was supplemented with 10 µl of 5 mg/ml MTT. After a 3 h incubation, the cells were lysed with 100 µl per well of extraction buffer (20% SDS/N,N-dimethylformamide, pH 4.7), and further incubated overnight. The absorbance at 570 nm was measured (Microplatereader, SLT, Salzburg, Austria). Wells without cells were used as blanks.

**Analysis** Intracellular GSH level was determined by high-performance liquid chromatography using ammonium 7-fluorobenzo-2-oxa-1,3-diazole-4-sulfonate as fluorogenic reagent according to the method of Toyo’oka and Imai. MT content was determined by using a modified ²⁰³Hg-binding assay. Glutathione peroxidase activity (GSH-Px) was measured by the method of Lawrence and Burk using tert-butylhydroperoxide (0.3 mM) as a substrate. Catalase activity and superoxide dismutase (SOD) activity were measured by the method of Wheeler et al. and by the method of Imanari et al., respectively. Protein concentration was determined by Lowry’s method.

**Animal study** Five-week-old female nude mice (Crl:CD-I(ICR)-nu) were kindly supplied by Charles River Japan, Inc., Atsugi. Nude mice were maintained under specific-pathogen-free conditions with free access to water and diet. A group of nude mice were inoculated subcutaneously into their backs with HeLa/MTH cells (1×10⁷ cells of HeLa/MT7 or HeLa/MT27/mouse). Two weeks after the inoculation of HeLa/MTH cells, tumor-bearing nude mice were randomized into control and experimental groups with four mice per group. A group of nude mice was injected intraperitoneally either with cis-DDP (15 µmol/kg) or saline.

The antitumor activity was evaluated twice a week by measuring tumor volume according to a Battelle Columbus Laboratories protocol. For each tumor, perpendicular diameters were recorded and tumor volumes (v) were calculated using the formula

\[ v = \frac{ab^2}{2} \]

where a is the maximal diameter of the tumor and b is the diameter at right angles to a. Further, tumor weights were measured 3 weeks after the injection of cis-DDP. MT content in the transplanted tumors was determined using the ²⁰³Hg-binding assay as modified by us as modified by us²⁵ and 2 and 5 weeks after the inoculation of HeLa/MTH cells.

**Statistical calculations** The data were analyzed by means of Student’s t test.

**RESULTS**

**Preparation and characterization of HeLa cells expressing mouse MT-I** To express mouse MT-I in HeLa cells, a cDNA which corresponds to only the coding region of mouse MT-I mRNA was used, eliminating any effect of regulatory elements which might exist in its 5’ or 3’ noncoding region. This cDNA fragment was synthesized by a method involving PCR and inserted into the HindIII-BglII site of the pSV2 vector. In this construct (pSV2MT-I), as shown in Fig. 1, mouse MT-I cDNA is expressed in a HeLa cell line.

**Fig. 1.** Structure of plasmid pSV2MT-I designed for expression of mouse MT-I. A β-globin cDNA in the expression vector pSV2-β-globin was replaced by mouse MT-I cDNA as described in “Materials and Methods.”

**Fig. 2.** MT contents in HeLa/MTH cell lines. MT levels in HeLa S3 cells, control cell lines and HeLa/MTH cell lines were determined as described in “Materials and Methods.” Each value of MT level is the mean±SD obtained from four experiments.
expressed under the control of the SV40 early promoter, which is considered to be a strong promoter in HeLa cells. Human HeLa cells were transfected with pSV2MT-I together with a pSV2gpt vector and were selected for mycophenolic acid resistance, in which the background level is thought to be lower compared with G418 selection. About 200 mycophenolic acid-resistant colonies were isolated. Of 64 isolates measured for MT content as described in "Materials and Methods," 27 expressed greater MT levels than the parental cells. Seven cell lines were established by limiting dilution from one clone which expressed the highest MT level among the 27 isolates, and were examined for MT content under standard culture conditions. Seven transformants (HeLa/MTH; HeLa/MT6, HeLa/MT7, HeLa/MT12, HeLa/MT17, HeLa/MT21, HeLa/MT26 and HeLa/MT27) were selected and used for further analysis. As shown in Fig. 2 and Table I, the MT content of these HeLa/MTH cell lines was 4–20-fold higher than that of the parental HeLa S3 cells. No significant change in MT level in either transformant was detected even after the cells were maintained in the culture medium for 2 months without mycophenolic acid. The elevated MT content in the transformants might be due to clonal variation resulting from heterogeneity in the cell population. To exclude this possibility, we established 2 mycophenolic acid-resistant cell lines transfected with pUC119 and pSV2gpt, and designated them HeLa/C1 and HeLa/C2. As shown in Table I and Fig. 2, MT levels in these cell lines were similar to those of the parental HeLa S3 cells. These results strongly suggest that the elevated MT content in HeLa/MTH cell lines resulted from the MT cDNA introduced into the cells.

To establish these seven HeLa/MTH cell lines as genuine transformants, high-molecular-weight DNAs from each of the HeLa/MTH cell lines, two control cells and parent HeLa S3 cells were isolated. Southern blot analysis of these genomic DNAs digested with EcoRI is shown in Fig. 3. Multiple strong signals were observed in all of the HeLa/MTH cell lines by probing with a mouse MT-I cDNA fragment. This observation indicates that HeLa/MTH cell lines have a number of copies of mouse MT cDNA in multiple sites of the genome. The patterns and intensities of these bands were very similar among these HeLa/MTH cell lines. This observation is consistent with the assumption that all these cell lines were derived from a single mycophenolic acid-resistant clone (as originally described above). In the case of control cell lines and parent HeLa S3 cells, a few faint bands around 9.4 kbp were detected. These signals should be due to endogenous human MT genes, since the mouse MT-I cDNA probe was capable of detecting human MT DNAs under our normal conditions.

Table I. Activities of Antioxidant Enzymes and Glutathione Content in HeLa/MTH Cell lines

|          | Catalase (HCHO nmol/µg protein) | GSH-Px (U/mg protein) | SOD (U/mg protein) | GSH (mmol/mg protein) |
|----------|---------------------------------|-----------------------|--------------------|-----------------------|
| HeLaS3   | 0.70±0.06                       | 18.36±1.06            | 7.75±0.76          | 13.74±0.60            |
| HeLa/C1  | 0.78±0.31                       | 25.49±2.60            | 7.49±0.38          | 12.94±1.77            |
| HeLa/C2  | 0.91±0.39                       | 46.58±2.24            | 5.97±0.89          | 11.97±0.67            |
| HeLa/MT6 | 0.54±0.12                       | 27.61±1.48            | 7.37±0.51          | 14.85±3.16            |
| HeLa/MT7 | 0.98±0.05                       | 18.98±0.67            | 8.77±0.25          | 12.68±0.39            |
| HeLa/MT12| 0.62±0.09                       | 44.99±5.32            | 7.49±0.38          | 13.99±1.37            |
| HeLa/MT17| 0.63±0.11                       | 20.03±2.37            | 8.38±0.38          | 25.02±4.11            |
| HeLa/MT21| 0.74±0.07                       | 32.60±5.18            | 6.73±0.38          | 19.99±2.47            |
| HeLa/MT26| 0.54±0.18                       | 19.87±2.94            | 8.13±0.76          | 12.91±3.05            |
| HeLa/MT27| 0.62±0.09                       | 26.72±8.56            | 8.38±0.38          | 18.59±0.91            |
hybridization conditions. In fact, the probe did detect endogenous human MT mRNA, as shown in Fig. 4. These data clearly showed that multiple copies of mouse MT-I cDNA were stably introduced into multiple loci of the genome of HeLa cells.

To determine the mRNA level in the cells, total RNA was extracted from each cell line and was analyzed by northern blotting with mouse MT-I cDNA as a probe. As expected, strong bands at signal II, which corresponds to transcripts of mouse MT-I cDNA carried by pSV2MT-I vector, were found in all HeLa/MTH cells, whereas no band at the signal II position was detected in control cells. The levels of endogenous human MT mRNA in each HeLa/MTH cell line (signal I) were similar to those of the parental HeLa S3 cells. The transcript of mouse MT-I cDNA contains extra sequences derived from the pSV2 vector, so that the size of signal II is about 500 nucleotides longer than that of the endogenous human MT mRNA indicated by signal I in Fig. 4. Indeed, only the transcripts of endogenous human MT genes (signal I) were accumulated when HeLa/MTH cells were incubated in the presence of 10 µM Cd or 100 µM Zn overnight, without any accumulation of mouse MT mRNA indicated by signal II (data not shown). These data clearly showed that mouse MT-I cDNA incorporated into the genome of HeLa cells was transcribed efficiently to produce MT mRNA in the absence of a metal inducer. The 32P-radioactivities in signal I and signal II in the autoradiogram in Fig. 4A were quantitated by an image analyzer (Fig. 4B). Relative MT mRNA levels accumulated in cells were roughly correlated with MT content.

In addition to MT content, intracellular GSH level and the activities of catalase, GSH-Px and SOD as antioxidant enzymes were determined in HeLa/MTH cell lines and in control cells. As shown in Table I, there were no significant alterations in the levels of these factors among these cell lines. The growth rate in cells overproducing mouse MT-I was not significantly different from that of control cells (data not shown).

Resistance of HeLa/MTH cell lines to cis-DDP The sensitivity of MT-overproducing cell lines and control cells to cis-DDP was examined by MTT assay as described in “Materials and Methods.” Survival curves of two HeLa/MTH cell lines (HeLa/MT7 and HeLa/MT27), control cells (HeLa/C1) and parental HeLa S3 cells are shown in Fig. 5. There was no increase in the survival rate of the HeLa/MTH cell lines on treatment with cis-DDP. The parental HeLa cells and control cell lines (HeLa/C1) were slightly more resistant than HeLa/MTH cell lines to cis-DDP. There was no correlation between the degree of resistance and the increase in the cellular level of MT.

![Fig. 4. Northern blot analysis of MT mRNA in HeLa/MTH cell lines.](image)

![Fig. 5. Relative cell survival of HeLa/MTH cell lines after treatment with cis-DDP.](image)
Animal study To study the role of MT in resistance to cis-DDP in vivo, two MT-overproducing HeLa cell lines, HeLa/MT7 and HeLa/MT27, were inoculated subcutaneously into nude mice. Tumor MT contents of HeLa/MT7 (66.7±11.2 nmol Hg bound/g) and HeLa/MT27 (75.2±14.3 nmol Hg bound/g) cells increased 3-fold compared with that of control HeLa/C1 cells (22.9±5.2 nmol Hg bound/g) 2 weeks after the inoculation of these transformants (at the time of cis-DDP injection). The elevated MT concentrations in these tumors were maintained for at least 5 weeks after the inoculation (data not shown).

The changes in volume of tumors with varying levels of MT after cis-DDP injection are shown in Fig. 6. Growth of the tumor derived from HeLa/C1 cells was almost completely inhibited by cis-DDP injection. However, tumors derived from both HeLa/MT7 and HeLa/MT27 cells showed marked resistance to cis-DDP. The tumor weights of mice transplanted with control HeLa/C1 cells determined at 21 days after the injection of 15 µmol/kg of cis-DDP were approximately 20% of those untreated with cis-DDP, while antitumor activity of cis-DDP was not observed in the mice transplanted with tumors derived from HeLa/MT7 and HeLa/MT27 cells (Fig. 7).

DISCUSSION

Several in vivo studies have shown that the elevation of MT level in tumors results in an increase in resistance to cis-DDP. The MT-mediated cis-DDP resistance of tumors can be overcome by administration of propargylglycine (PPG), a specific inhibitor of the cystathionein pathway, which suppresses Zn-mediated MT induction in tumors. However, Zn might produce multiple effects on the cells, and cause various effects other than MT induction in vivo. To investigate the actual contribution of MT to the acquisition of resistance to cis-DDP in vivo and in vitro, we introduced mouse MT-I cDNA under the control of SV40 promoter into HeLa cells, expecting constitutive MT expression in the absence of a heavy metal inducer. Stable transformants (HeLa/MTH) thus constructed were confirmed to carry multi-copies of mouse MT-I cDNA in their chromosomal DNA and to produce 4–20-fold larger amounts of MT than the parent HeLa cells. There is a possibility that a large amount of metal-free apoprotein is accumulated in HeLa/MTH cell lines due to a deficiency of cellular heavy metals such as zinc. To examine this possibility, the culture medium was supplemented overnight with 5 µM Zn, which does not cause any induction of endogenous MT, and no elevation of MT level was observed in either transformant. This indicates that transcripts of mouse MT-I cDNA contribute to the production of functional mouse MT-I protein in these cells rather than the production of apoprotein.

In in vitro experiments, these MT-overproducing cell lines showed significant Cd resistance as compared with their parent cells and there was a good correlation between the degree of Cd resistance and the cellular MT level (data not shown). This indicated that the exogenous mouse MT-I gene produced a functional MT protein in human HeLa cell transformants. However, these HeLa/MTH cell lines did not show any resistance to cis-DDP and seemed to be more sensitive than the control cells. These results seemed to be consistent with the report by Koropatnick and Pearson. They established and used MT-I-overexpressing CHO cells transfected with the pBR322 vector carrying mouse MT-I cDNA without any eukaryotic promoter sequences. HeLa/MTH cell lines established here had low plating efficiency, as they reported, although a corre-
lation between the level of MT and plating efficiency was not observed (data not shown). There have been several transfection studies using an extrachromosomally replicating bovine papilloma virus-based vector expressing human MT-IIA at high level in the cells.26–30 Among these studies, Kelley et al. reported that the transformants with high levels of MT showed increased resistance to cis-DDP.27 However, Schilder et al. reported that the same transformant, which Kelley et al. had established, showed a similar cis-DDP sensitivity to that of the parental cell lines.30 The reasons for this inconsistency in MT-related resistance to cis-DDP are not clear at present. It is possible, however, that resistance to cis-DDP is attributable to many biological processes, which are easily influenced by multiple factors such as differences in methodology, transformants, and levels of MT.

To evaluate the role of MT in cis-DDP resistance in vivo, two independent HeLa cell lines overproducing mouse MT-I (HeLa/MT7 and HeLa/MT27) were inoculated into nude mice. The resultant tumors contained 3-fold higher levels of MT compared with those derived from control cell lines (HeLa/C1) and clearly showed increased resistance to cis-DDP. This is the first evidence that mouse tumors derived from transformants with high MT levels have cis-DDP resistance in vivo. These transformants showed increased MT production without change of their GSH contents and activities of antioxidant enzymes.

These results strongly indicate that an elevated MT level confers resistance to cis-DDP in vivo, even when it does not provide resistance in vitro. The reason for this discrepancy between the in vivo and in vitro efficacy of MT as a cis-DDP resistance factor requires further study. Elucidation of the molecular mechanism may provide a molecular basis for creating more efficient methods to screen anticancer drugs.

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REFERENCES

1) Rosenberg, R. C., Van Camp, L., Trosko, J. E. and Mansour, V. H. Platinum compounds: a new class of potent antitumor agents. Nature, 222, 385–386 (1969).

2) Krakoff, I. H. and Lippman, A. J. Clinical trials of cis-platinum diaminodichloride in patients with advanced cancer. In “Recent Results in Cancer Research,” ed. G. Mathe and R. K. Old, pp. 185–193 (1974). Springer-Verlag, Berlin and New York.

3) Swelling, L. A. and Kohn, K. W. Mechanism of action of cis-dichlorodiammineplatinum (II). Cancer Treat. Rep., 63, 1439–1444 (1979).

4) Andrews, P. A. and Hoxell, S. B. Cellular pharmacology of cisplatin: perspectives on mechanisms of acquired resistance. Cancer Cells, 2, 35–43 (1990).

5) Andrews, P. A., Sriharasha, V., Mann, S. C. and Howell, S. B. Cis-diaminedichloroplatinum (II) accumulation in sensitive and resistant human ovarian carcinoma cells. Cancer Res., 48, 68–73 (1988).

6) Basu, A. and Lazo, J. S. A hypothesis regarding the protective role of metallothioneins against the toxicity of DNA interactive anticancer drugs. Toxicol. Lett., 50, 123–135 (1990).

7) Lazo, J. S. and Basu, A. Metallothionein expression and transient resistance to electrophilic antineoplastic drugs. Semin. Cancer Biol., 2, 267–271 (1991).

8) Masada, H., Ozols, R. E., Lai, G. M., Fojo, A., Rothenberg, M. and Hamilton, T. C. Increased DNA repair as a mechanism of acquired resistance to cis-diaminedichloroplatinum (II) in human ovarian cancer cell lines. Cancer Res., 48, 5713–5716 (1988).

9) Kägi, J. H. R. and Schaffer, A. Biochemistry of metallothionein. Biochemistry, 27, 8509–8515 (1988).

10) Endresen, L., Bakka, A. and Rugstad, H. E. Increased resistance to chlorambucil in cultured cells with a high concentration of cytoplasmic metallothionein. Cancer Res., 43, 2918–2926 (1983).

11) Cagen, S. Z. and Klaassen, C. D. Binding of glutathione-depleting agents to metallothionein. Toxicol. Appl. Pharmacol., 54, 229–237 (1980).

12) Thornally, P. J. and Vasak, M. Possible role for metallothionein in protection against radiation-induced oxidation stress. Kinetics and mechanism of its reaction with superoxide and hydroxyl radicals. Biochim. Biophys. Acta, 827, 36–44 (1985).

13) Abel, J. and Ruiter, N. Inhibition of hydroxy-radical generated DNA degradation by metallothionein. Toxicol. Lett., 47, 191–196 (1989).

14) Satoh, M., Naganuma, A. and Imura, N. Metallothionein induction prevents toxic side effects of cisplatin and adriamycin used in combination. Cancer Chemother. Pharmacol., 21, 176–178 (1988).

15) Naganuma, A., Satoh, M. and Imura, N. Prevention of lethal and renal toxicity of cis-diaminedichloroplatinum (II) by induction of metallothionein synthesis without compromising its antitumor activity in mice. Cancer Res., 47, 983–987 (1987).

16) Kondo, Y., Satoh, M., Imura, N. and Akimoto, M. Effect of bismuth nitrate given in combination with cis-diaminedichloroplatinum (II) on the antitumor activity and renal toxicity of the latter in nude mice inoculated with human bladder tumor. Cancer Chemother. Pharmacol., 29, 19–23 (1991).
Naganuma, A., Satoh, M. and Imura, N. Specific reduction of toxic side effects of adriamycin by induction of metallothionein in mice. *Jpn. J. Cancer Res.*, **79**, 406–411 (1988).

Satoh, M., Naganuma, A. and Imura, N. Involvement of cardiac metallothionein in prevention of adriamycin induced lipid peroxidation in the heart. *Toxicology*, **53**, 231–237 (1988).

Satomi, N., Sakurai, A., Haranaka, R. and Haranaka, K. Preventive effect of several chemicals against lethality of recombinant human tumor necrosis factor. *J. Biol. Response Mod.*, **7**, 54–64 (1988).

Satoh, M., Miura, N., Naganuma, A., Matsuzaki, N., Kawamura, E. and Imura, N. Prevention of adverse effects of γ-ray irradiation by metallothionein induction by bismuth subnitrate in mice. *Eur. J. Cancer Clin. Oncol.*, **25**, 1727–1731 (1989).

Satoh, M., Kloth, D. M., Kadhim, S. A., Chin, J. L., Naganuma, A., Imura, N. and Cherian, M. G. Modulation of both cisplatin nephrotoxicity and drug resistance in murine bladder tumor by controlling metallothionein synthesis. *Cancer Res.*, **53**, 1829–1832 (1993).

Endresen, L., Schjerven, L. and Rugstad, H. E. Tumors from a cell strain with a high content of metallothionein show enhanced resistance against cis-dichlorodiammineplatinum. *Acta Pharmacol. Toxicol.*, **55**, 183–187 (1984).

Bakka, A., Endresen, L., Johnsen, A. B. S., Edminson, P. D. and Rugstad, H. E. Resistance against cis-dichlorodiammineplatinum in cultured cells with a high content of metallothionein. *Toxicol. Appl. Pharmacol.*, **61**, 215–226 (1981).

Kasahara, K., Fujisawa, Y., Nishino, K., Ohmori, T., Sugimoto, Y., Komiya, K., Matuda, T. and Saijo, N. Metallothionein content correlates with the sensitivity of human small cell lung cancer cell lines to cisplatin. *Cancer Res.*, **51**, 3237–3242 (1991).

Andrews, P. A., Murphy, M. P. and Howell, S. B. Metallothionein-mediated cisplatin resistance in human ovarian carcinoma cells. *Cancer Chemother. Pharmacol.*, **19**, 149–154 (1987).

Lohrer, H. and Robson, T. Overexpression of metallothio- nein in CHO cells and its effect on cell killing by ionizing radiation and alkylating agents. *Carcinogenesis*, **10**, 2279–2284 (1989).

Kelley, S. L., Basu, A., Teicher, B. A., Hacker, M. P., Hamer, D. H. and Lazo, J. S. Overexpression of metallothionein confers resistance to anticancer drugs. *Science*, **241**, 1813–1815 (1988).

Kainas, B., Lohrer, H., Karin, M. and Herrlich, P. Overexpressed human metallothionein II A gene protects Chinese hamster ovary cells from killing by alkylating agents. *Proc. Natl. Acad. Sci. USA*, **87**, 2710–2714 (1990).

Lohrer, H., Robson, T., Grindley, H., Foster, S. and Hall, A. Differential effects on cell killing in metallothionein overexpressing CHO mutant cell lines. *Carcinogenesis*, **11**, 1937–1941 (1990).

Schilder, R. J., Hall, L., Monks, A., Handel, L. M., Fornace, A. J. J., Ozols, R. F., Fojo, A. T. and Hamilton, T. C. Metallothionein gene expression and resistance to cisplatin in human ovarian cancer. *Int. J. Cancer*, **45**, 416–422 (1990).

Koropatnick, J. and Pearson, J. Altered cisplatin and cadmium resistance and cell survival in Chinese hamster ovary cells expressing mouse metallothionein. *Mol. Pharmacol.*, **44**, 44–50 (1993).

Minamino, T., Nomura, M., Tamai, M., Moritani, S., Ohshima, T. and Miyamoto, K. In vivo cisplatin resistance of rat ascites hepatoma AH66. *Cancer Lett.*, **108**, 153–156 (1996).

Durnam, D. M., Perrin, F., Gannon, F. and Palmiter, R. D. Isolation and characterization of the mouse metallothionein-I gene. *Proc. Natl. Acad. Sci. USA*, **77**, 6511–6515 (1980).

Gorman, C. High-efficiency gene transfer into mammalian cells. In “DNA Cloning—A Practical Approach,” ed. D. M. Glover, pp. 143–190 (1985). IRL Press, Oxford.

Chen, C. and Okayama, H. High-efficiency transformation of mammalian cells by plasmid DNA. *Mol. Cell. Biol.*, **7**, 2745–2752 (1987).

Mulligan, R. C. and Berg, P. Selection for animal cells that express the *Escherichia coli* gene coding for xanthine-guanine phosphoribosyltransferase. *Proc. Natl. Acad. Sci. USA*, **78**, 2072–2076 (1981).

Maniatis, T., Fritsch, E. F. and Sambrook, J. “Molecular Cloning: A Laboratory Manual” (1982). Cold Spring Harbor Laboratory, New York.

Davis, L. G., Dibner, M. D. and Battey, J. F. Formaldehyde gel for electrophoretic separation of RNA and Northern blot. In “Basic Methods in Molecular Biology,” ed. L. G. Davis, M. D. Dibner and J. F. Battey, pp. 143–146 (1986). Elsevier, New York.

Niks, M. and Otto, M. Towards an optimized MTT assay. *J. Immunol. Methods*, **130**, 149–151 (1990).

Toyo’oka, T. and Imai, K. High-performance liquid chromatography and fluorometric detection of biologically important thiols, derivatized with ammonium 7-fluororhodamine-2-oxa-1,3-diazole-4-sulphonate (SBD-F). *J. Chromatogr.*, **262**, 495–500 (1983).

Kotsonis, F. N. and Klaassen, C. D. Comparison of methods for estimating hepatic metallothionein in rats. *Toxicol. Appl. Pharmacol.*, **42**, 583–588 (1977).

Wheeler, C. R., Salzman, J. A., Elsayed, N. M., Omaye, S. T. and Korte, D. W. J. Automated assay for superoxide dismutase, catalase, glutathione peroxidase, and glutathione reductase activity. *Anal. Biochem.*, **184**, 193–199 (1990).

Imanari, T., Hirota, M., Miyazaki, M., Hayakawa, K. and Tamura, Z. Improved assay method for superoxide dismutase. *J. Clin. Exp. Med.*, **101**, 496–497 (1977).

Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.*, **193**, 265–275 (1951).

Ovejera, A. A., Houchens, D. P. and Barker, A. D. Chemo-therapy of human tumor xenographs in genetically athymic mice. *Ann. Clin. Lab. Sci.*, **8**, 50 (1978).

Mulligan, R. C. and Berg, P. Expression of a bacterial gene in mammalian cell. *Science*, **209**, 1422–1427 (1980).