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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 antineoplastic 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 agents[10, 11] and scavenging free radicals.[12, 13] It has been demonstrated that the toxic effects of *cis*-DDP,[14–16] adriamycin (ADR),[14, 17, 18] tumor necrosis factor[19] and γ-irradiation[20] 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 Pearson[31] 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

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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′-GCGAATTCAAGCTTCTGCAGCCGCCAUGGACCCCAACTGC-3′, an antisense primer, 5′-GCGAATTCGGATCCCTGCAGTTATCAGGCACAGCACGTGCA-3′, with 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 *Hin*dIII-*Bam*HI 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*.[33] This *Hin*dIII-*Bam*HI cDNA fragment was inserted into the *Hin*dIII-*Bgl*II site of a plasmid vector pSV2-β-globin[34] 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 (1×10^6 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.[35] After 12 h transfection, the cells were rinsed, refed with growth medium and then incubated at 37°C for 24 h. A total of 1×10^6 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/μl, Sigma).[36] MT-overproducing transformants (HeLa/MTH cell lines) were established by limiting dilution and designated HeLa/MT6, 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 procedure[37] followed by at least two cycles of CsCl equilibrium density gradient centrifugation, since *E. coli* proteins are extremely toxic to animal cells.

Southern blot analysis High-molecular-weight DNAs prepared from 7×10^7 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 *Eco*RI, separated by electrophoresis in 0.8% agarose gels and transferred to nitrocellulose filters. The blots were hybridized with ^{32}P-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 ^{32}P-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 1×10^7 cells of each cell line using the guanidium 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 glyoxal[37] 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.[39] Cells (1×10^4 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.[40] MT content was determined by using a modified ^{203}Hg-binding assay.[15, 41] Glutathione peroxidase activity (GSH-Px) was measured by the method of Lawrence and Burk using *tert*-butylhydroperoxide (0.3 m*M*) as a substrate. Catalase activity and superoxide dismutase (SOD) activity were measured by the method of Wheeler *et al.*[42] and by the method of Imanari *et al.*,[43] respectively. Protein concentration was determined by Lowry's method.[44]

Animal study Five-week-old female nude mice (Crj: CD-1(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 (1×10^7 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 Battele Columbus Laboratories protocol.[45] For each tumor, perpendicular diameters were recorded and tumor volumes (v) were calculated using the formula

$$v\ (\text{mm}^3) = ab^2/2,$$

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 ^{203}Hg-binding assay[41] as modified by us[15] 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 *Hin*dIII-*Bgl*II site of the pSV2 vector.[34] In this construct (pSV2MT-I), as shown in Fig. 1, mouse MT-I cDNA is

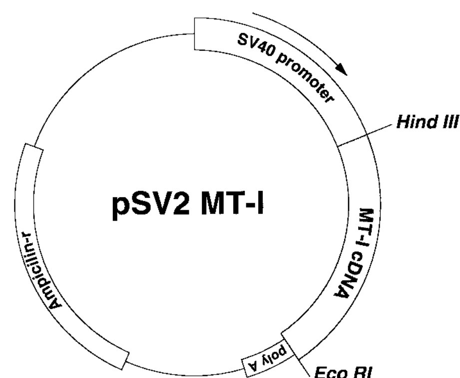

Fig. 1. Structure of plasmid pSV2MT-I designed for expression of mouse MT-I. A β-globin cDNA in the expression vector pSV2-β-globin was replaced by mouse MT-I cDNA as described in "Materials and Methods."

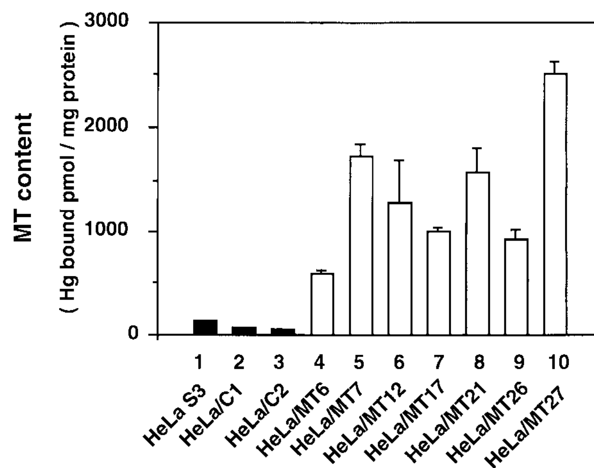

Fig. 2. MT contents in HeLa/MTH cell lines. MT levels in HeLa S3 cells, control cell lines and HeLa/MTH cell lines were determined as described in "Materials and Methods." Each value of MT level is the mean±SD obtained from four experiments.

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±060
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	18.03±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

expressed under the control of the SV40 early promoter, which is considered to be a strong promoter in HeLa cells.[46] 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.[36] 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 *Eco*RI 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

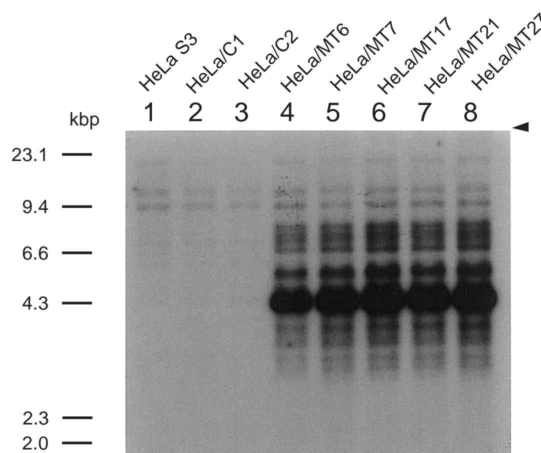

Fig. 3. Southern blot analysis of genomic DNAs of HeLa/MTH cell lines. High-molecular-weight DNA from HeLa S3 cells (lane 1), control cell lines (lanes 2 and 3) and each of the HeLa/MTH cell lines (lanes 4–8) was digested with *Eco*RI and analyzed by Southern blot hybridization. Mouse MT-I cDNA fragment was used as a probe. Molecular weight markers were prepared by *Hin*dIII digestion of λ phage DNA, and the positions are indicated on the left side of the figure.

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 ^{32}P-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.

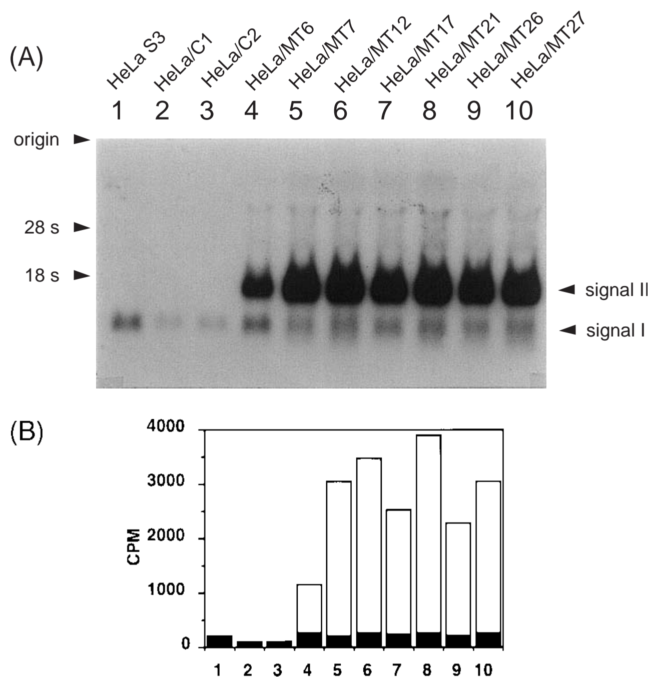

Fig. 4. Northern blot analysis of MT mRNA in HeLa/MTH cell lines. (A) Total RNA was extracted from HeLa S3 cells (lane1), control HeLa cell lines (lanes 2 and 3) or HeLa/MTH cell lines (lanes 4–10) and analyzed by northern blot hybridization. Mouse MT-I cDNA fragment was used as a probe. Positions of the origin of agarose gel electrophoresis, and 28S and 18S human rRNA are shown by arrowheads. (B) ^{32}P-radioactivity in signals I (■) and II (□) in autoradiogram (A) was quantitated by an image analyzer. Signal I and signal II indicate endogenous MT mRNA in HeLa cells and pSV2MT-I-derived MT mRNA, respectively.

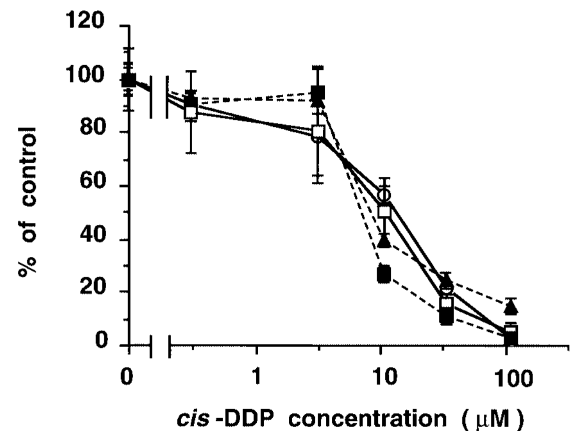

Fig. 5. Relative cell survival of HeLa/MTH cell lines after treatment with *cis*-DDP. The sensitivity of HeLa S3 (○), HeLa/C1 (□), HeLa/MT7 (▲) and HeLa/MT27 (■) cells to various concentrations of *cis*-DDP was measured by MTT assay as described in "Materials and Methods." Values are the mean±SD (bars) obtained from at least four experiments.

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).

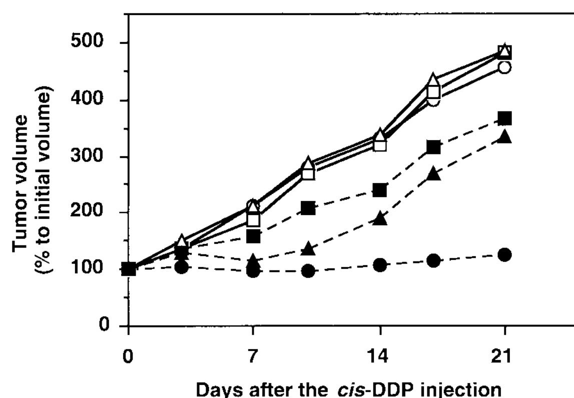

Fig. 6. Effect of *cis*-DDP on the growth of mouse tumor derived from MT-overproducing HeLa cells. Volumes of tumors derived from HeLa/C1 (●, ○), HeLa/MT7 (■, □) and HeLa/MT27 (▲, △) were calculated after the tumor-bearing mice were treated with (●, ■, ▲) or without (○, □, △) *cis*-DDP injection. The values are mean±SD for four mice.

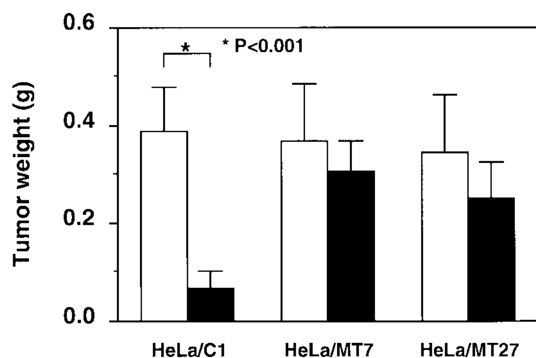

Fig. 7. Antitumor activity of *cis*-DDP in mice inoculated with MT-overproducing HeLa cells. Tumor weights of saline-treated (□) or *cis*-DDP-treated (■) nude mice were determined 3 weeks after the injection of *cis*-DDP. The values are mean±SD for four mice.

DISCUSSION

Several *in vivo* studies have shown that the elevation of MT level in tumors results in an increase in resistance to *cis*-DDP.[21, 22, 32] 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.[21] 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.[31] They established and used MT-I-overexpressing CHO cells transfected with the pBR322 vector carrying mouse MT-I cDNA without any eukaryotic promoter sequences.[31] 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 *in vitro* 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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