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Induction of Metallothionein Isoforms in Rat Hepatoma Cells by Various Anticancer Drugs[1]

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The induction of metallothionein (MT) isoforms (MT-1, -2) by anticancer drugs was investigated in cultured rat hepatoma H4 II E C3 cells. The steady-state expression of MT-1 mRNAs was higher than that of MT-2 mRNAs. During incubation of the cells with various anticancer drugs, namely, adriamycin, epirubicin, *cis*-diamminedichloroplatinum(II) (CDDP), and *cis*-diammine(1,1-cyclobutyldicarboxylato)platinum(II), both MT-1 and MT-2 mRNAs were coordinately inducible: the levels of isoMT mRNA reached a maxim of approximate by 6-fold at 3 h. Immunofluorescent studies revealed that the cytosolic fluorescence in the cells exposed to 1 μM CDDP for 48 h was more intensified than that in the untreated cells. Transfer of antisense oligonucleotides resulted in marked reduction of isoMT mRNA, and upon exposure to 5 μM CDDP for 48 h, the viabilities of these cells dropped to 25.8% of the controls. These results indicate that anticancer drugs are potent inducers of MT isoforms in hepatoma cells and that a decrease in cellular MTs enhances the susceptibility of hepatoma cells to CDDP. Thus, we conclude that endogenous MTs play a role in determining the sensitivity or resistance of cancer cells to clinically important anticancer agents.

Key words: anticancer drug, drug resistance, metallothionein, metallothionein isoform, rat hepatoma cell.

Metallothioneins (MTs) are a family of low molecular weight, cysteine-rich proteins that bind heavy metals such as zinc (Zn^{2+}), copper (Cu^{2+}), and cadmium ions (Cd^{2+}) (*1-3*). MTs have been purified from a broad range of species and many different tissues. In mammals, there are two major isoMTs, designated MT-1 and MT-2, and the characteristic structures of their primary amino acids have been confirmed by molecular cloning and sequence analyses of cDNAs and genes (*4-6*). Consequently, recent genetic studies also confirm the evidence that the primary structures of isoMTs have been well conserved during evolution (*6, 7*).

The most remarkable biological feature of MTs is their inducibility. Injection of animals with heavy metals causes MTs to accumulate in the liver and kidney (*5, 6, 8*). Cells that have been selected for Cd^{2+} resistance produce higher than normal levels of MTs (*4, 9*). These and earlier studies have well documented the roles of MTs in homeostasis of the essential metals and detoxification of cytotoxic heavy metals (*2, 3, 7, 8*). Besides heavy metals, biosynthesis of MTs can be induced by a variety of factors: pathophysiological stress, hormones, cytokines (*2-5, 7, 8*), oxidative stress (*10*), and X-ray irradiation (*11*), as well as such clinically used anticancer drugs as *cis*-diamminedichloroplatinum(II) (CDDP) (*12*), chlorambucil (*13*), adriamycin (ADM), and bleomycin (*12*). Previous observations suggest that an increase in cellular MTs can afford protection against the toxic actions of anticancer drugs, and overexpression of MTs may be involved in the cellular resistance or sensitivity to some anticancer drugs (*12-18*).

Hepatoma is generally insensitive and relatively resistant to treatment with anticancer drugs or radiation when compared to other solid tumors such as medulloblastoma of the brain, small cell lung carcinoma, some urogenital cancers, and malignant lymphomas (*19*). Development of the resistance of hepatoma to anticancer drugs has been explained by several mechanisms (*20*). However, it is also noteworthy that hepatoma can produce a considerable amount of MTs, because the liver, the origin of the hepatoma, is one of major organs which physiologically synthesizes both MT-1 and MT-2 (*1-3, 6, 14*), and that high cellular concentration of MTs in hepatoma may be a factor in the resistance to anticancer agents. From this point of view, several questions remain unanswered: (a) which type of isoMTs is preferentially produced by hepatoma cells? (b) which isoMTs are overexpressed in hepatoma cells upon exposure to anticancer drugs? (c) what mechanism(s) are involved in the putative induction of cellular MTs? (d) are increased MTs isoform-specific or selective functions? and (e) the final and key point, do MT

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Abbreviations: DMEM, Dulbecco's modified Eagle's medium; G3PDH, glyceraldehyde-3-phosphate dehydrogenase; MT, metallothionein; PBS, phosphate-buffered saline.

isoforms contribute to protection of hepatoma against clinically used anticancer drugs? To elucidate these points, we have used a rat hepatoma cell line that expresses two isoMTs, developed two DNA probes which respectively hybridize to rat isoMT mRNAs, and employed them in Northern hybridization analysis. We report here the induction of MT-1 and MT-2 mRNAs and their proteins in cultured rat hepatoma cells (*23*) upon exposure to anticancer drugs, and we demonstrate that a decrease in MT synthesis by antisense oligonucleotide transfer can confer cellular sensitivity to CDDP.

MATERIALS AND METHODS

Anticancer Drugs, Enzymes, and Other Chemicals—*Cis*-diamminedichloroplatinum(II) (CDDP) and *cis*-diammine-(1,1-cyclobutyldicarboxylato)platinum(II) (CBDCA) were gifts from Bristol Meyers Squibb (Brussels, Belgium), adriamycin (ADM) and epirubicin (EPR) were gifts from Farmitalia Carlo Erba (Tokyo). Restriction endonucleases, *Eco*81I and *Bbi*II, were obtained from Takara Shuzo (Shiga). 5′-[α-^{32}P]dCTP (3,000 Ci/mmol) was from Amersham (Arlington Heights, IL) and partial G3PDH cDNA was from Clontech (Palo Alto, CA). Mouse monoclonal anti-MT antibody (DAKO-MT, E9) and fluorescent isothiocyanate conjugated anti-mouse IgG were purchased from Dako (Carpinteria, CA) and American Qualex (La Miranda, CA), respectively. Lipofectin was supplied by Life Technologies (Basel, Switzerland), and AlamarBlue was purchased from Alamar BioSciences (Sacramento, CA). All other chemicals used were of analytical grade.

DNA Probes Specific to Rat MT Isoforms—To amplify the partial rat isoMT cDNAs by PCR, three oligonucleotide primers were synthesized on an Applied Biosystem Model 381A DNA synthesizer (Foster city, CA). The upstream 20-mer primer (5′-ATGGACCCCAACTGCTCCTG-3′) was identical in sequence to the 5′-terminal coding regions (nucleotide number 1 to 20, the numbering of nucleotide sequences starts at adenine residue in the initiation codon) of both rat MT-1 (*6*) and MT-2 cDNAs (data from GenBank, accession #11794). A downstream 13-mer (5′-AGA-ATAAACAGGC-3′) complementary to nucleotide position 308-320 of the MT-1 cDNA and 14-mer (5′-TAATCAAG-TCAAGT-3′) complementary to 312-325 of the MT-2 cDNA were chosen by comparing the 3′ non-coding regions of MT-1 and MT-2 cDNAs. PCR was carried out using a combination of the upstream 20-mer primer and one of the two downstream primers (13-mer or 14-mer), and λ gt 11 rat liver cDNA library (Clontech, Palo Alto, CA) as template. PCR conditions were 94°C for 30 s, 45°C for 1 min, and 72°C for 30 s for 30 cycles. PCR products were digested with *Eco*81I or *Bbi*II and analyzed on 2% agarose gel (*21*). The smaller restriction fragments of the *Eco*81I or *Bbi*II digests were extracted from the gel using a Prep-A-Gene DNA purification matrix (Bio-Rad, Richmond, CA), verified by DNA sequencing, and designated probe 1 (P-1) for MT-1 and probe 2 (P-2) for MT-2, respectively.

These probes and partial G3PDH cDNAs were ^{32}P-labeled using Megaprime DNA labeling systems (Amersham, Arlington Heights, IL) in accordance with the manufacturer's directions. The ^{32}P-labeled probes each had a specific radioactivity of 5.0×10^9 dpm/μg DNA and were used in Southern and Northern hybridization analyses (*21, 22*).

Cell Culture and Extraction of Total RNA—Rat hepatoma H4 II E C3 cells (ATCC CRL 1600) (*23*) were maintained in DMEM supplemented with 10% heat-inactivated fetal bovine serum (Gibco, Paisley, UK), penicillin G (100 units/ml), and streptomycin (100 μg/ml). Hepatoma cells were incubated in this standard medium at 37°C for 24 h under a semihumidified atmosphere containing 5% CO_2 prior to the experimental treatments. Cells were washed with serum-free DMEM before use in the present studies.

Total cellular RNA was prepared by the guanidine isothiocyanate method (*21, 24*). The yield, purity, and integrity of the extracted RNA were determined spectrophotometrically (*22, 25*).

Northern Blot Analysis and Quantitative Determination of isoMT mRNAs—Total RNAs (6 μg) were electrophoresed on a 1.2% agarose gel under denaturing conditions, then transferred onto nylon filters for hybridization with ^{32}P-labeled probe P-1 or P-2. Hybridization filters were washed, then exposed to Kodak XAR-5 X-ray film (Rochester, NY) as described (*22, 24*).

Expressions of isoMT mRNAs in H4 II E C3 cells were quantitatively determined by slot-blot analysis. In brief, 6-μg portions of total RNAs were slot-blotted onto a nylon membranes and hybridized with ^{32}P-labeled P-1 or P-2. The amounts of isoMT mRNA expression were quantified by scanning the hybridization intensity in the slot on the resulting autoradiogram (BAS-2000, Fuji Photofilm, Tokyo). Data obtained were normalized with the amount of G3PDH mRNAs as the internal standard (*23*), and the accumulation of isoMT mRNAs in the cells was defined as the increase of signal intensity in treated cells relative to that in untreated cells.

Oligonucleotides and Transfection—Phosphorothioate sense (5′-ATGGACCCCAACTGC-3′) oligonucleotides, which were identical to nucleotide position 1 to 15 of both rat isoMT cDNAs, and antisense (5′-GCAGTTGGGGTCC-AT-3′) oligonucleotides were synthesized on a DNA synthesizer (model 381A, Applied Biosystem), detritylated and desalted in accordance with the manufacturer's directions, and checked for purity by reversed-phase high-performance liquid chromatography.

Rat hepatoma H4 II E C3 cells (0.5×10^4) were cultured to confluency in 96-well plates (Becton Dickinson Labware, Lincoln Park, NJ) under the standard conditions, rinsed with serum-free DMEM, and transfected with phosphorothioate sense or antisense oligonucleotides (5 μM) using Lipofectin (Life Technologies, Basel, Switzerland) (*26*).

Cytotoxicity Assay—Transfected cells were maintained for 5 h in the standard media, rinsed with serum-free DMEM, then incubated with CDDP in serum-free DMEM for an additional 48 h. The viabilities of the treated cells were then measured by AlamarBlue assay (Alamar BioSciences, Sacramento, CA) with minor modification (*27*). In brief, cells in 96-well plates were kept at 37°C for an additional 3 h with one-tenth volume of AlamarBlue dye. Absorption at 570 and 600 nm in each well and in the blank wells was determined by use of a microplate reader model MTP-120 (Corona Electric, Ibaraki), and the viabilities of the cells were calculated following the manufacturer's formula (*27*).

Indirect Immunofluorescence Microscopy—The expression of MT proteins in rat hepatoma H4 II E C3 cells was

visualized by the indirect immunofluorescence method. Cell samples maintained on glass cover slips were permealized with PBS containing 0.1% Triton X-100 and washed with PBS containing 0.05% Tween 20. Nonspecific binding of the first antibody was blocked by incubation with fetal calf serum (2% in PBS) for 60 min. The samples were then incubated with anti-MT antibody, E9 (IgG_1, Kappa, DAKO, Carpinteria, CA) (*28*) at a working dilution of 1/100. Nonimmune immunoglobulins were used at the same concentration to provide controls for specificity. The secondary antibody was goat anti-mouse IgG conjugated with fluorescent isothiocyanate (American Qualex, LaMirada, CA) diluted 1:100 in 2% FCS in PBS. Preparations were subsequently washed in PBS, mounted in 50% glycerol in PBS containing *p*-phenylenediamine (Sigma Chemical, St. Louis, MO) (*29*), and examined by fluorescence microscopy, using the procedures previously described (*30*).

Statistical Analysis—All data obtained by densitometric or spectrophotometric analyses were processed, and the statistical significance of the differences was assessed by Student's *t* test.

RESULTS

Detection of Rat isoMT mRNAs with Isoform-Specific Probes P-1 and P-2—To synthesize the isoform-specific DNA probes, partial cDNA clones of MT-1 (nucleotide number 1-320) and MT-2 (1-319) were amplified from the rat liver cDNA library by PCR. Figure 1 shows the electrophoretic analysis of the PCR products and their restriction fragments on the agarose gel. The combined use of the upstream 20-mer primer and one of two downstream primers (13-mer for MT-1 or 14-mer for MT-2) in each case yielded a product of approximate by 320 bp (Fig. 1, lanes 1 and 3). The electrophoretic mobilities of the PCR products were in good agreement with the sizes expected from rat MT-1 and MT-2 cDNA sequences (320 bp for MT-1 and 319 bp for MT-2, respectively). Digestion of PCR products with an appropriate restriction endonuclease resulted in discrete fragments of 190 and 130 bp (Fig. 1, lanes 2 and 4). The 130 bp restriction fragments were extracted from the gel and designated as probes P-1 and P-2, respectively.

The specificities of probes P-1 and P-2 were assessed by Southern blot hybridization analysis. As shown in Fig. 2, ^{32}P-labeled P-1 hybridized to partial MT-1 cDNAs (nucleotide number 1-320), and did not cross-hybridize to partial MT-2 cDNAs (Fig. 2A); and probe P-2 hybridized to partial MT-2 cDNAs (1-319) and not to partial MT-1 cDNAs (Fig. 2B). These results demonstrate that the synthetic DNA probes P-1 and P-2 are respectively specific to rat MT-1 and MT-2 cDNAs.

Expression of isoMT mRNAs in Rat Hepatoma Cells—Expressions of isoMT mRNAs in rat hepatoma H4 II E C3 cells were determined by Northern hybridization analysis. A single band occurred at appropriately 400 bases (Fig. 3) corresponding to MT-1 and MT-2 mRNAs. Although the radioactivities of ^{32}P-P-1 and ^{32}P-P-2 were similar (5.0×10^9 dpm/μg DNA), more RNA reacted with ^{32}P-P-1 in untreated hepatoma cells than with ^{32}P-P-2 (Fig. 3, lanes 1 and 3). These results indicate that untreated H4 II E C3 cells express both MT-1 and MT-2 mRNAs, and that the basal level of MT-1 mRNA expression is higher than that of MT-2. Treatment of H4 II E C3 cells with 5 μM ADM

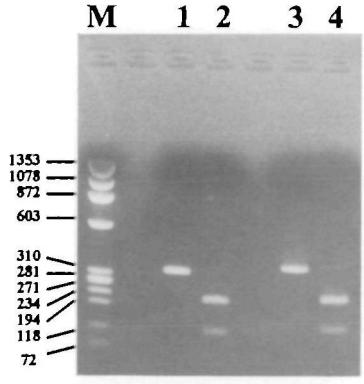

Fig. 1. **Agarose gel electrophoresis of the PCR products and their restriction digests.** PCR products and their restriction digests were analyzed on 2% agarose gel in the presence of ethidium bromide. Lane 1, PCR products generated by the combination of the upstream 20-mer primer (5′-ATGGACCCCAACTGCTCCTG-3′, identical to the 5′-terminal coding regions of both rat isoMT cDNAs) and downstream 13-mer (5′-AGAATAAACAGGC-3′, complementary to nucleotide position 308-320 of the MT-1 cDNA) (*6*); lane 2, *Eco*81I restriction digests of the PCR products shown in lane 1; lane 3, PCR products generated by the combination of the upstream 20-mer primer and another downstream 14-mer primer (5′-TAATCAAGTC-AAGT-3′, complementary to nucleotide position in 312-325 of the MT-2 cDNA, data from GenBank, accession #11794); lane 4, *Bbi*II digests of the PCR products shown in lane 3; lane M, size markers from Hae III digests of the ϕX-174 phage DNA.

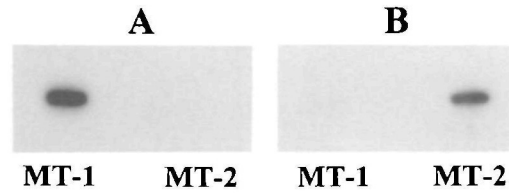

Fig. 2. **Specificities of the DNA probe P-1 and P-2.** Partial cDNAs (5 ng) of MT-1 and MT-2 were amplified by PCR, slot-blotted to nylon membranes, and hybridized with ^{32}P-labeled probe P-1 (A) or P-2 (B). Southern hybridization was carried out as described in "MATERIALS AND METHODS."

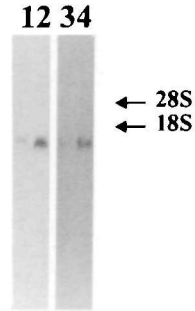

Fig. 3. **Northern blot analysis of isoMT mRNAs in rat hepatoma cells treated with ADM.** Total cellular RNA was extracted from 1×10^6 rat hepatoma H4 II E C3 cells incubated at 37°C for 3 h in the absence (lanes 1 and 3) or presence (lanes 2 and 4) of 5 μM ADM in serum-free DMEM. The expression of isoMT mRNAs was detected by Northern blot analysis with isoform-specific probe P-1 (lanes 1 and 2) or P-2 (lanes 3 and 4) as described in "MATERIALS AND METHODS."

resulted in increases in both MT-1 and MT-2 mRNAs (Fig. 3, lanes 2 and 4). Densitometric quantification of the hybridization signals revealed that amounts of MT-1 and MT-2 mRNAs in ADM-treated cells were 4.2- and 6.0-fold greater than those in untreated cells, respectively.

Induction of isoMT mRNAs in Rat Hepatoma Cells Exposed to Anticancer Drugs—Treatment of rat hepatoma cells with anticancer drugs resulted in the induction of both MT-1 and MT-2 mRNAs (Fig. 4). By exposure of hepatoma cells to ADM or EPR, the levels of isoMT mRNA expressions began to rise within 1–2 h, reached a maximum of approximate by 6-fold at 3 h, then gradually returned to the basal levels within 24 h (Fig. 4, A and B). The profiles of isoMT mRNA inductions in hepatoma cells treated with CDDP or CBDCA (Fig. 4, C and D) were generally similar to those of cells treated with ADM or EPR (Fig. 4, A and B). The expression of G3PDH mRNAs was not changed by any antineoplastic agents during observation (data not shown).

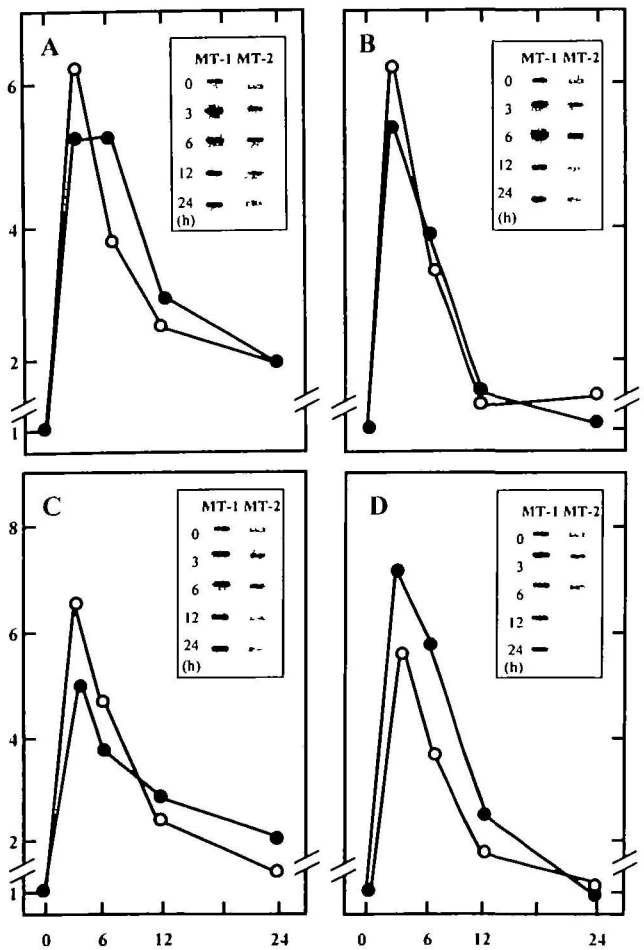

Fig. 4. **Time course of isoMT mRNA expressions in rat hepatoma cells treated with anticancer drugs.** Rat hepatoma H4 II E C3 cells (1×10^6) were incubated at 37°C with 5 μM ADM (A), 5 μM EPR (B), 1 μM CDDP (C), or 5 μM CBDCA (D) in serum free DMEM. At the indicated times, total RNAs were extracted from the cells and slot-blotted onto a nylon filter. The expressions of MT-1 (●) and MT-2 (○) mRNAs were respectively detected with isoform-specific probe P-1 or P-2 (inset) and semiquantified as described in "MATERIALS AND METHODS." Data are the mean of four experiments.

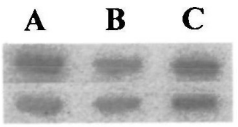

Fig. 5. **Expression of isoMT mRNAs in rat hepatoma cells transfected with oligonucleotides.** 1 μg of total RNAs extracted from rat hepatoma H4 II E C3 cells transfected without oligonucleotides (A), transfected with antisense oligonucleotides (B), or with sense oligonucleotides (C) were independently slot-blotted to a nylon membrane and hybridized-probed simultaneously with both ^{32}P-labeled P-1 and P-2 (upper panel). Expression of G3PDH mRNAs in these cells was also determined as experimental control (lower panel).

Expression of isoMT mRNAs in Rat Hepatoma Cells Transfected with Oligonucleotides—Expressions of isoMT mRNAs in rat hepatoma cells transfected with oligonucleotides were determined by slot-blot analysis. As shown in Fig. 5, the amount of isoMT mRNAs in hepatoma cells transfected with sense oligonucleotides was similar to that in the control cells transfected without oligonucleotides. On the other hand, the expression of isoMT mRNAs in the cells transfected with antisense oligonucleotides was markedly reduced compared to the control cells. The amounts of G3PDH mRNAs in the cells transfected with or without oligonucleotides were not changed.

Expression of MT Proteins in Rat Hepatoma Cells—The indirect immunofluorescence technique was employed to detect MT proteins in rat H4 II E C3 hepatoma cells. We used mouse monoclonal antibody (Dako-MT, E9) raised against horse MTs, which reacts with both rat MT-1 and MT-2 (*28*). Faint immunofluorescence was observed in the cytosol of untreated H4 II E C3 cells (Fig. 6A), and cytosolic fluorescence in the cells incubated with 1 μM CDDP for 48 h was increased (Fig. 6B). Upon exposure to 5 μM CDDP for 48 h, the immunofluorescence in hepatoma cells transfected with oligonucleotides was also intensified (Fig. 7). However, the number of immunoreactive cells transfected with antisense oligonucleotides (Fig. 7B) was less than that of cells transfected with sense oligonucleotides (Fig. 7A).

Cytotoxic Effect of CDDP on Rat Hepatoma Cells Transfected with Oligonucleotides—Viability of rat hepatoma cells exposed to CDDP was quantitatively estimated by an AlamarBlue assay. As shown in Fig. 8, H4 II E C3 cells transfected with sense or antisense oligonucleotides appeared to be viable for 48 h in the absence of CDDP. A cytotoxic effect of CDDP was observed when the hepatoma cells were incubated for 48 h in the presence of 5 μM CDDP. The viability of the cells transfected with sense oligonucleotide was reduced to 66.2% of the control cells, although this reduction was not statistically significant compared to that of the non-transfected controls (62.8%). On the other hand, the viability of H4 II E C3 cells transfected with antisense oligonucleotides was reduced to 25.8% of the control cells in the presence of 5 μM CDDP for 48 h.

DISCUSSION

Mammalian MTs exhibit many of the features characteristic of multigene families (*4, 7, 8*). Although several pseudogenes have been described (*4, 8*), rats and mice are thought to synthesize only two functional isoMTs, MT-1,

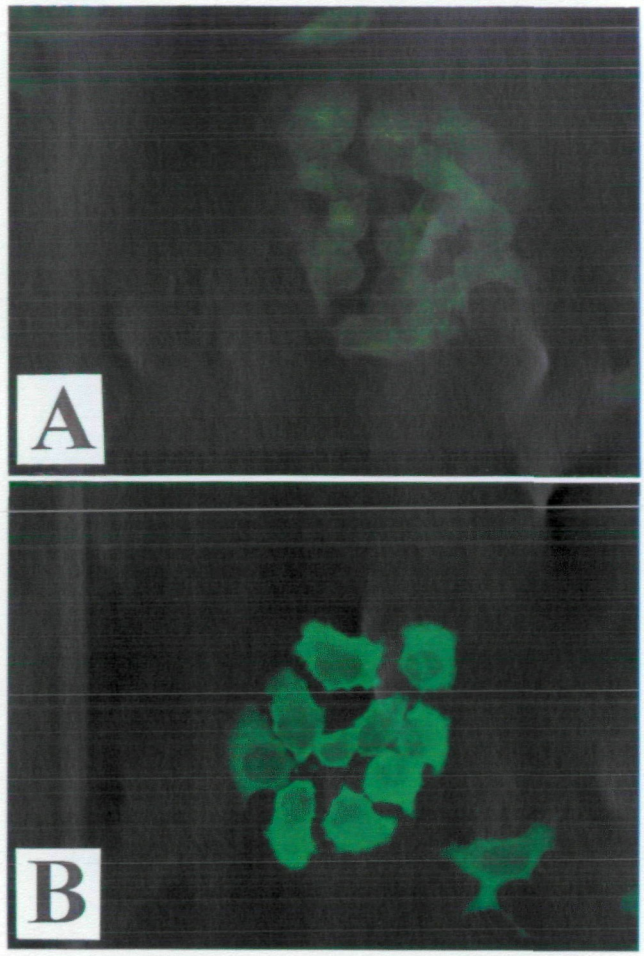

Fig. 6. **Indirect immunofluorescent micrographs of rat hepatoma cells exposed to CDDP.** Rat hepatoma H4 II E C3 cells on glass cover slips were incubated at 37°C for 48 h in the absence (A) or presence of 1 μM CDDP (B) in serum-free DMEM. Expression of MT proteins in hepatoma cells was examined by indirect immunofluorescence as described in "MATERIALS AND METHODS."

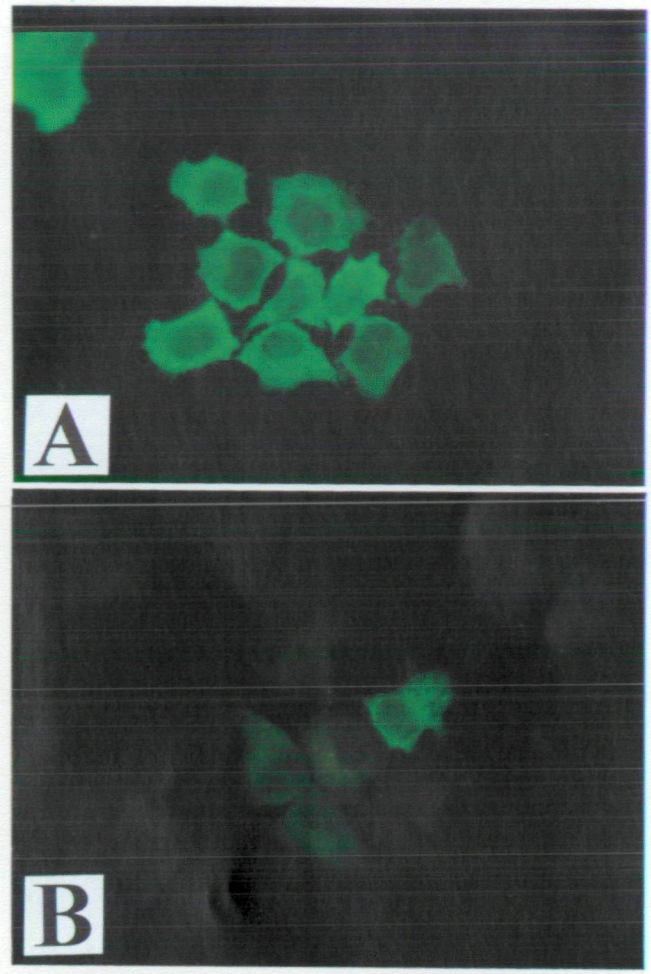

Fig. 7. **Indirect immunofluorescent micrographs of rat hepatoma cells transfected with oligonucleotides following exposure to CDDP.** Rat hepatoma H4 II E C3 cells on glass cover slips were preincubated under the standard conditions, then transfected with phosphorothioate sense (A) or antisense (B) oligonucleotides using Lipofectin (*26*). Transfected cells were kept incubating for an additional 48 h with 5 μM CDDP in serum-free DMEM. Expression of MT proteins in the cells was investigated by indirect immunofluorescence as described in "MATERIALS AND METHODS."

and MT-2 (*5, 6, 9*). As predicted from the primary amino acid structures, the coding sequences of the cloned isoMT cDNAs are highly conserved and highly homologous (*7, 8*). In rats, for example, the homology in the coding regions between MT-1 and MT-2 cDNAs amounts to 83%. Studies utilizing oligonucleotide probes capable of discriminating between isoMT mRNA species will provide important information about the gene expressions of specific isoMTs. Accordingly, we synthesized two specific DNA probes, P-1 and P-2, by PCR following digestion with restriction endonuclease. Sequencing of these probes revealed that their nucleotide sequences were identical to the 3′ partial regions of the respective isoMT cDNAs (*6*), and nucleotide homology between P-1 and P-2 was less than 43%. As predicted from these data, it was confirmed that the two synthesized DNA probes hybridized to specific sequences in the respective isoMT cDNAs and were powerful tools for quantitative analyses of isoMT gene expressions by Northern and dot-blot hybridizations. By using these probes, we first defined that both MT-1 and MT-2 mRNAs in rat hepatoma cells were coordinately inducible by anticancer drugs, anthracyclines and Pt-coordination complex.

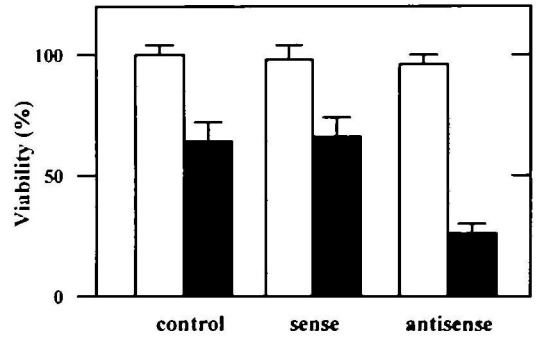

Fig. 8. **Viability of rat hepatoma cells exposed to CDDP.** Rat hepatoma H4 II E C3 cells transfected without oligonucleotides (control), or with sense or antisense oligonucleotides were incubated for 48 h in the absence (open bar) or presence (closed bar) of 5 μM CDDP in serum-free DMEM. Viabilities of the cells were then measured by AlamarBlue assay as described in "MATERIALS AND METHODS." Viability of the control cells incubated in the absence of CDDP is presented as 100%, and data are mean ± SD of four experiments.

The present study showed that both MT-1 and MT-2 mRNAs were expressed in untreated rat hepatoma H4 II E C3 cells. The basal level of MT-1 mRNA was higher than that of MT-2 mRNA and the magnitude of increase of MT-1 mRNA by anticancer drugs was similar to that of MT-2 mRNA. These data indicate that MT-1 is preferentially inducible over MT-2 in H4 II E C3 hepatoma cells exposed to anticancer drugs. Differential expression of isoMTs was also reported in human hepatoblastoma cells (*31*), growing neonatal cells and mouse tumor cells (*32*), and embryonal carcinoma cells (*33*). Although the mechanisms regulating the differential expression of isoMT genes are unknown (*8, 34*), such differential expression probably reflects the origin of the cell line, the history of exposure to heavy metals and various other inducers, and isoform-specific activation of basal regulatory elements existing in the 5′ flanking region of isoMT genes (*5, 7, 8, 31*).

We first examined the time-course of induction of isoMTs in hepatoma cells upon exposure to anticancer drugs: Pt-coordination complexes (CDDP and CBDCA) and anthracyclines (ADM and EPR). The increases of isoMT mRNAs caused by these drugs were transient and returned almost to the basal level within 24 h. In contrast, cells exposed to Cd^{2+} accumulated high levels of MT-1 and MT-2 mRNAs 80- to 120-fold the basal level(s), which did not return to the basal levels by 24 h (data not shown). These findings suggest that anticancer drugs are also potent inducers of MT synthesis in rat hepatoma cells by increasing the amounts of isoMT transcripts, but that they induce expression of the isoMT genes by a different mechanism to that of heavy metals.

The immunofluorescence study showed that MTs in the hepatoma cells were induced by CDDP and that the hepatoma cells transfected with antisense oligonucleotides expressed less MTs than the cells transfected with or without sense oligonucleotides. We also demonstrated that the effect of CDDP on cytotoxicity of rat hepatoma cells transfected with antisense oligonucleotides was much more potent than that of the cells transfected with or without sense oligonucleotides. These results are similar to those of Kondo *et al.* (*35*), who reported that embryonic fibroblast cells from transgenic mice with targeted disruptions of both MT-1 and MT-2 genes showed enhanced sensitivity to anticancer drugs such as CDDP. Thus, the inducibility of MTs in the cells is an important factor in determining their sensitivity to lethal cytotoxicity of clinically used anticancer agents.

Chemotherapy is generally less effective against hepatomas than other solid tumors. The resistance of hepatomas to anticancer drugs has been explained by several factors, such as MTs (*12*), P-glycoprotein (*20*), and DNA repair (*36*). Bahnson *et al.* (*37*) reported that bladder tumors expressing low levels of MTs were more likely to show a complete pathological response following chemotherapy with such drugs as CDDP, methotrexate, and vinblastine than those expressing high levels of MTs. However, they showed that there was no relationship between the expression level of P-glycoprotein in the tumors and the response to chemotherapy (*37*). In the present study, we confirm that (a) hepatoma cells in the steady-state express MTs, especially MT-1, (b) both isoMTs in hepatoma cells are induced temporarily by anticancer drugs by a different mechanism from that of induction by heavy metals, and (c) MTs in the cells are important factors in determining the sensitivity of the cells to lethal cytotoxicity of clinically used anticancer drugs. Therefore, we conclude that a relatively high level synthesis of endogenous MTs is responsible for the poor response of hepatoma to CDDP and other anticancer drugs, and that induced synthesis of MTs in the cells may be partly responsible for the acquired resistance of hepatomas to some anticancer chemotherapies. In order to establish clinical strategies to overcome the poor response and acquired resistance of hepatomas to chemotherapeutic agents, we need to clarify further the determinants of the drug-resistant phenotype *in vivo* as well as *in vitro* studies using cultured cells.

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