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Altered Cisplatin and Cadmium Resistance and Cell Survival in Chinese Hamster Ovary Cells Expressing Mouse Metallothionein

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SUMMARY

Metallothionein (MT) proteins are associated with resistance to the toxic effects of heavy metals, chemotherapeutic drugs, and alkylating agents. It has been suggested that MT may mediate both resistance to toxic agents and cellular metal homeostasis. To study the role of MT, we obtained cells expressing a range of MT levels in the absence of heavy metal induction. We co-transfected the eukaryotic G418 resistance vector pSV2*neo* and mouse MT-1 cDNA in a pBR322 vector into Chinese hamster ovary cells. Of 200 transfected clonal cell populations, five had constitutive MT expression ranging from 31 to 87 ng of MT/mg of protein. All five populations had increased resistance to cadmium but were less resistant to cisplatin than control cells. On the other hand, the level of foreign MT expression correlated well with the degree of cisplatin resistance among the five clones. Resistance to ionizing radiation and growth rate in the absence of drug or radiation treatment were not affected. However, transfected MT gene expression inhibited the ability of Chinese hamster ovary cells to form colonies in the absence of toxic drug treatment ($r = -0.95$). The perturbation of cisplatin sensitivity after genetic alteration of MT expression indicates a role for MT in drug resistance: however, the fact that transfected MT gene expression decreased rather than increased drug resistance and decreased plating efficiency in the absence of drug implies that the role of MT may not be one of simply "scavenging" toxic molecules. These data suggest a role for MT in homeostatic cellular processes that, when disturbed by transfection of active MT genes, have an effect on cellular drug resistance.

MTs are low molecular mass (<10 kDa) metal-binding proteins involved in resistance to toxic heavy metals and metal homeostasis (1, 2). They are encoded by a family of genes in primates (a single functional MT-2 gene, at least five functional MT-1 genes, and a brain-specific MT-3 gene) (3) and rodents (single copies of MT-1, MT-2, and MT-3 genes). MT genes are induced by heavy metals and a variety of other agents (4–7) and have been implicated as mediators of resistance to many toxic agents; cells selected on the basis of resistance to cadmium and zinc are cross-resistant to the toxic effects of cisplatin, chlorambucil, prednimustine (8), mitomycin C, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (9), and, in some cases, ionizing radiation (8). Conversely, human tumor cell lines selected for resistance to cisplatin may (10) or may not (11) have elevated levels of MT or increased cadmium resistance [reviewed by Basu and Lazo (12)]. Cells expressing human MT-II_A at high levels by virtue of transformation with a BPV eukaryotic expression vector containing the MT gene were resistant to melphalan, chlorambucil, and cisplatin (10), as well as *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine and methylnitrosourea (13, 14), but not ionizing radiation (10, 11). However, correlation of resistance to toxicity with MT expression under constitutive eukaryotic transcriptional control, at levels comparable to those observed after maximal induction with heavy metals, has not been reported. The following question remains: is there cellular drug resistance associated with lower level MT expression (on the order of that induced in cells after treatment with heavy metals) from genes under the control of eukaryotic cellular promoters? To address this question, we transfected mMT-1 cDNA sequences (without ligated eukaryotic promoters) into CHO cells to observe constitutive MT expression at physiological levels in the absence of heavy metal induction or viral transformation. We describe the construction of a panel of CHO cell lines expressing variable amounts of mMT by virtue of transfection of mMT-1 cDNA in the pBR322 shuttle vector, which lacks efficient eukaryotic transcriptional control sequences. As a consequence, expression of integrated mMT-1 cDNA depended heavily upon control elements in CHO DNA. mMT-1 mRNA and protein were expressed in <3% of successfully transfected cells (i.e., five of 200 transfected clonal populations). When cells expressing foreign MT were compared with controls, four of five mMT-expressing cells were more

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ABBREVIATIONS: MT, metallothionein; mMT, mouse metallothionein; CHO, Chinese hamster ovary; bp, base pair(s); RIA, radioimmunoassay; BPV, bovine papillomavirus.

sensitive to cisplatin than control cells transfected with vectors alone. Although there was no correlation between the level of foreign MT expression and the degree of increased sensitivity to cisplatin in CHO cells, foreign MT expression correlated well with resistance to cadmium, indicating that MT expressed from incorporated mouse cDNA was functional and its site of insertion did not preclude the ability of cells to express a resistant phenotype. On the other hand, foreign MT expression was inversely correlated with cellular plating efficiency in the absence of cisplatin. Sensitivity to the toxic effects of ionizing radiation and the growth rate in the absence of drug or radiation treatment were unaffected by expression of foreign MT genes. We conclude that perturbation of both drug resistance and cellular homeostasis occurs in cells with genetically altered MT biosynthesis. This suggests that the cellular role of MT during treatment with toxic agents other than heavy metals is not simple "scavenging" of reactive toxic molecules. Rather, MT is likely involved in processes that are important in normal cell function and that also mediate cisplatin resistance.

Materials and Methods

Cell lines and culture conditions. CHO cells were grown in α minimal essential medium (GIBCO/BRL) supplemented with L-glutamine (GIBCO) and 26 mM sodium bicarbonate, 50 μg/ml gentamycin, 250 ng/ml fungizone, 25 mM cysteine, and 10% fetal bovine serum (all from Sigma).

Polybrene-mediated gene transfer and cell selection. DNA was transfected into CHO cells by a modification of the Polybrene-mediated method of Cheney *et al.* (15) and Aubin *et al.* (16). Cells (2×10^6) were treated with a mixture of (a) the pBR322 plasmid with a full length MT-1 cDNA [400 bp, including 16 bp of pBR322 DNA at the 3′ end, inserted between the *Eco*RI and *Hind*III cloning sites; the sequence contained 132 bp of 3′ untranslated region and a 3′ poly(A) tail in addition to the entire coding region of MT-1] (17) and (b) the eukaryotic expression vector pSV2*neo* (18), at a molar ratio of 10:1 (recombinant pBR322:pSV2*neo*), to increase the probability of concomitant incorporation of MT-1 cDNA with the pSV2*neo* vector. Control CHO cells were transfected with a mixture of pSV2*neo* and nonrecombinant pBR322 DNA. A total of 5 μg of DNA and Polybrene (10 μg/ml; Abbot Laboratories) were applied to each plate of 5×10^5 cells, and cells were incubated for 6–8 hr. Cells were then exposed to 15% dimethylsulfoxide for 4 min, washed, exposed to nonselective medium for 24–48 hr, and placed in medium containing Geneticin (400 μg/ml G418, active form; GIBCO/BRL) to select colonies expressing *neo*. Two hundred G418-resistant colonies were picked from cells co-transfected with pBR322/mMT-1 cDNA/pSV2*neo* vector DNA, four G418-resistant colonies were picked from cells co-transfected with pSV2*neo* and pBR322 without the MT-1 cDNA, and three G418-sensitive colonies were picked from parental CHO cells. Selected cell populations were maintained for 1 week of 4 in 400 μg/ml G418 sulfate and were assessed for mMT-1 cDNA incorporation, mMT-1 mRNA accumulation, MT protein levels, and cellular resistance to cisplatin, cadmium, and ionizing radiation.

DNA analysis and MT mRNA measurement. DNA and total cellular RNA were isolated by standard methods (19, 20). DNA was cleaved with *Eco*RI and analyzed by Southern blotting (19) and hybridization to radiolabeled mMT-1 cDNA isolated from the pBR322 vector, to assess the presence of mMT-1 cDNA in high molecular weight CHO DNA. In some cases, DNA was dot blotted (20) and hybridized, in sequence, to mMT-1 cDNA and radiolabeled mouse satellite DNA isolated from liver hepatoma cells (21) as described (20). This provided a measure of relative amounts of incorporated mMT-1 cDNA in CHO cell lines. The mouse satellite DNA readily hybridized to CHO cell DNA under the conditions used. Hybridization was carried out as described previously (20), for 16 hr at 65° in 0.45 M NaCl, 10× Denhardt's solution, 100 μg/ml yeast tRNA, 10 mg/ml poly(A), 0.1% sodium dodecyl sulfate, 0.1% sodium pyrophosphate, 10% dextran sulfate, and 80 μg/ml denatured herring sperm DNA, followed by washing for 2.5 hr at 65° in 0.45 M NaCl and for 1 hr at 65° in 0.15 M NaCl. Total cellular RNA was analyzed by separation on denaturing formaldehyde/agarose gels, followed by transfer to Hybond nylon filters or immobilization on nylon filters by RNA dot blotting (22). Northern blots and dot blots were hybridized to the same mMT-1 cDNA under the same stringency conditions described above, to assess accumulation of mMT-1 mRNA. Rehybridization of the same filters to a β-actin cDNA probe (2.1 kilobases in pBR322) (23) provided comparative quantitation within the same sample of RNA. Regression analysis of a plot of probe hybridized versus micrograms of RNA dotted yielded values of MT-1 and β-actin mRNA accumulated (mean ± standard error), and MT-1 mRNA accumulation was expressed relative to β-actin mRNA accumulation.

MT protein measurement. MT was measured by a competitive solid-phase RIA as described previously (7, 24), using rabbit antiserum directed against rat MT-2 (25). The antibody cross-reacted with both human MT (25, 26) and mMT (7, 24). Although specific for MT, the RIA did not differentiate between MT-1 and MT-2.

Total protein content of cell samples was measured based on shifts in absorbance on binding of Coomassie blue (Bradford assay), using the technique described by the assay kit (Bio-Rad protein assay) manufacturer.

Cell survival measurement. Cells were harvested by trypsinization after growth to 80% confluence and were counted, and defined numbers of cells were plated in 60-mm tissue culture plates (five plates/cisplatin concentration) containing tissue culture medium with all supplements. The number of cells per plate was chosen to allow growth of 100–200 colonies and ranged from 200 cells for control plates to 2×10^5 cells for higher cisplatin concentrations.

Cisplatin treatment. After 24 hr to allow cell adhesion, medium was removed, cells were rinsed with ice-cold PBS, and fresh medium containing cisplatin (*cis*-dichlorodiammineplatinum; David Bull Laboratories, Melbourne, Australia) was added. Five plates of cells were treated with each drug concentration to allow assessment of error in each experiment. No further additions of drug were made during the course of colony formation. After growth at 37° for 10–14 days, colonies were stained with 1% methylene blue/1% glutaraldehyde in 70% ethanol and counted, and relative plating efficiency was correlated with drug concentrations. The D_{10} value [the concentration of drug that reduced the plating efficiency (survival) to 10% of control without drug] (27) was calculated for each cell population tested.

Cadmium treatment. After 24 hr to allow cell adhesion, fresh medium containing cadmium chloride was added. After 2 hr cadmium-containing medium was removed and fresh medium without cadmium was added. The short cadmium treatment was chosen in preference to continuous growth in metal-containing medium to minimize the influence of cadmium-induced CHO genes. Measurement of relative plating efficiency and D_{10} value was as described for cisplatin treatment. In some cases, control cells were induced for 24 hr with 37 μM $ZnCl_2$ before treatment with cadmium or cisplatin, to determine the effect of transient induction of constitutive MT genes on cisplatin, cadmium, and radiation resistance.

γ-Irradiation. Cells were irradiated with 1–10 Gy of γ-irradiation using a ^{60}Co source (Theratron Eldorado 6) at a dose rate of 1.60 Gy/min, as determined by calibration using an air ionization chamber (Capintec PR-06C) connected to an electrometer (Capintex 192X), 24 hr after plating in 60-mm tissue culture plates. Surviving colonies and D_{10} values were determined as for cisplatin survival.

Growth rate measurement. Duplicate samples of 1×10^5 cells were seeded in 6-cm plates in medium with or without 400 μg/ml G418 in the case of transfectants and without G418 in the case of CHO/P cells. The total amount of nucleic acids per plate was assessed by A_{260} measurement in 1% sodium dodecyl sulfate at 24-hr intervals, up to 96 hr.

Results

Production and characterization of CHO cells expressing mMT-1. Cell populations transfected with pSV2*neo* expressed G418 resistance at a frequency of 1:10^4. Of cells transfected with pBR322/mMT-1 cDNA in addition to pSV2*neo* (denoted CHO/mMT/*n*), only five of 200 G418-resistant clonal cell populations exhibited elevated MT mRNA expression in relation to CHO/*neo*/*n* cells (assessed by hybridization of the mMT-1 cDNA insert to RNA dot blots). Of the remaining 195 CHO/mMT/*n* cell populations, three of four (chosen at random) contained integrated mMT-1 cDNA sequences (assessed by hybridization of radiolabeled mMT-1 cDNA to DNA dot blots) (data not shown). The five cell populations expressing mMT-1 mRNA were chosen for further analysis. Four clonal populations of control G418-resistant cells transfected with pSV2*neo* and pBR322 (denoted CHO/*neo*/*n*) and three clonal populations of untransfected parental CHO cells (denoted CHO/P/*n*) were also chosen.

mMT-1 cDNA sequences were stably incorporated into high molecular weight DNA in the five CHO/mMT/*n* cell populations chosen (Fig. 1). DNA sequences from parent untransfected CHO cells grown in the absence of G418 (CHO/P/*n*) did not hybridize to the radiolabeled mMT-1 cDNA probe, but cells transfected with pSV2*neo* plus pBR322 without MT-1 cDNA insert (CHO/*neo*/*n*) showed a small amount of hybridization, presumably by association with the 16 bp of pBR322 sequences at the 3′ end of the hybridization probe. In DNA from cells transfected with both pSV2*neo* and pBR322 plus mMT-1 cDNA (CHO/mMT/*n*), *Eco*RI-derived DNA bands of greater intensity were evident. The incorporated MT-1 cDNA (with one *Eco*RI site at the boundary of the sequence) yielded multiple bands in CHO/mMT/6, CHO/mMT/1, and CHO/mMT/3 cells and a single band in CHO/mMT/2 and CHO/mMT/5 cells. DNA dot blots hybridized, in sequence, to the radiolabeled MT-1 cDNA probe and the mouse satellite DNA probe yielded mMT-1 cDNA:CHO β-actin DNA ratios (indicating relative amounts of MT-1 cDNA incorporation into genomic CHO cell DNA) of 0.8 (CHO/mMT/1), 1.0 (CHO/mMT/2), 0.8 (CHO/mMT/3), 0.6 (CHO/mMT/5), and 1.8 (CHO/mMT/6).

Incorporated mMT-1 cDNA was transcribed to produce MT mRNA in the absence of metal induction in the five of 200 G418-resistant clonal populations chosen for further analysis (Fig. 2; Table 1). Total cellular RNA analyzed by Northern blotting showed that three independent CHO/P clonal cellular

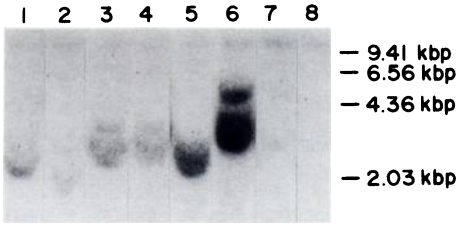

Fig. 1. Southern blot hybridization of *Eco*RI-digested high molecular weight DNA (10 μg/lane) isolated from clonal populations of CHO cells transfected with mMT-1 cDNA in pBR322 plus pSV2*neo* (*lane 1*, CHO/mMT/5; *lane 3*, CHO/mMT/3; *lane 4*, CHO/mMT/1; *lane 5*, CHO/mMT/2; *lane 6*, CHO/mMT/6). *Lane 2*, DNA from control cells transfected with pSV2*neo* plus pBR322 (CHO/*neo*/1); *lane 7*, untransfected CHO/P/1 cells; *lane 8*, untransfected CHO/P/2 cells. All lanes were run in the same gel, transferred to solid support, hybridized to radiolabeled probe (a purified [^{32}P]dCTP-labeled mMT-1 cDNA; *Eco*RI/*Hind*III insert from pBR322), and exposed to autoradiographic film at the same time. Lanes were cut out and ordered only to exclude noninformative samples.

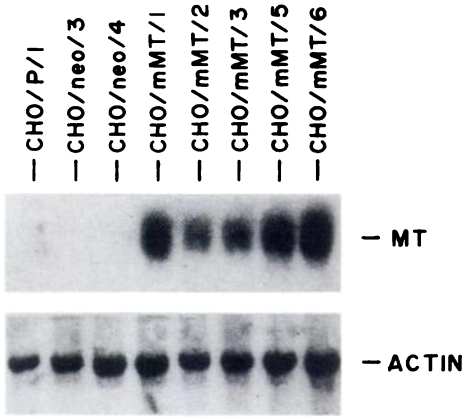

Fig. 2. Northern blot hybridization of total cellular RNA (5 μg/lane) isolated from control and mMT-expressing clonal CHO populations described in Materials and Methods and in the legend to Fig. 1. The same labeled MT-1 cDNA as described for Fig. 1 was used as hybridization probe. Rehybridization was performed on the same blot using a β-actin probe, to illustrate comparable amounts of RNA in each lane.

populations had no detectable MT mRNA expression (Table 1). The mMT-1 cDNA probe was capable of detecting CHO MT, because CHO/P/1 cells induced with zinc (37 μM $ZnCl_2$ for 4 hr) or cadmium (10 μM $CdCl_2$ for 4 hr) showed accumulated MT mRNA (Table 1). mMT-1 cDNA is 82% homologous to CHO MT-1 mRNA (28). Because only 65% homology exists between mMT-1 and CHO MT-2 (28), MT-2 sequences were unlikely to contribute to the hybridization signal. Different CHO/mMT/*n* clonal populations expressed MT-1 mRNA over a >2-fold range in the absence of heavy metal induction (Fig. 2; Table 1), with CHO/mMT/6 expressing the highest level of MT mRNA and CHO/mMT/3 the lowest, relative to CHO β-actin mRNA accumulation. CHO/*neo*/1 cells expressed a higher level of MT mRNA than did CHO/P cells, but accumulation was only 4–8% of that observed in CHO/mMT cells.

Transcribed mMT-1 mRNA was translated in CHO cells to produce immunologically detectable protein, as measured by a competitive solid-phase RIA that recognizes both CHO and mMT (Table 1) and binds MT-1 and MT-2 with similar affinities (24). MT protein levels correlated well with relative MT mRNA accumulation (r = 0.93) (Fig. 3) and were as high or higher than maximal inducible levels in CHO/P cells; CHO/mMT/3 and CHO/mMT/1, with the lowest levels of mMT-1, accumulated MT protein to approximately the same degree as did CHO/P cells maximally induced with zinc or cadmium. CHO/mMT/6 produced more than twice as much as MT-1 protein as did metal-induced CHO cells and 1.6 times as much as cadmium-induced B16F10 mouse melanoma cells (Table 1). CHO/P cells not treated with heavy metals had no detectable MT protein (i.e., they contained <2 ng/1 × 10^7 cells) (24).

Resistance of chimeric cells to cadmium. CHO/P, CHO/*neo*, and CHO/mMT clonal populations were tested for relative ability to form colonies in the presence of cadmium. Table 2 presents $D_{10(\text{cadmium})}$ values obtained with respect to MT protein level. There was no detectable increase in cadmium resistance due to transfection of control vectors, although a minor amount of MT became detectable. However, CHO cells expressing mMT had increased resistance to 2-hr cadmium treatment that correlated with MT level (r = 0.87). The short 2-hr cadmium treatment alone did not significantly increase MT accumulation or cadmium resistance in control cells (Table 2).

Resistance of chimeric cells to cisplatin. CHO/P, CHO/

TABLE 1

Characteristics of CHO cells expressing mMT-1

paragraph

	mMT-1 mRNA/ β-actin mRNA	MT	D_{10}[a]	
			Cisplatin	γ-Radiation
		ng/mg of protein	μM	Gy
Control cells				
CHO/P/1	ND[b]	ND	3.9, 4.8	5.5
CHO/P/2	ND	ND	4.5, 4.7	NM[c]
CHO/P/3	ND	ND	NM	NM
CHO/P/1 (Zn-induced)[d]	4.7 ± 0.4	37.3 ± 0.9	6.7, 7.3	5.3
CHO/P/1 (Cd-induced)[d]	6.9 ± 0.3	24.4 ± 1.0	NM	NM
CHO/*neo*/1	0.65 ± 0.01	3.3 ± 0.8	4.3, 4.8	5.0
CHO/*neo*/2	0.3 ± 0.2	2.8 ± 0.7	4.3, 4.8	NM
CHO/*neo*/3	0.4 ± 0.4	ND	4.5, 4.5	5.1
CHO/*neo*/4	0.2 ± 0.1	ND	NM	NM
Cells expressing mMT				
CHO/mMT/3	2.4 ± 0.2	31 ± 8	2.3 (2.1, 2.4)[e]	5.3
CHO/mMT/5	2.64 ± 0.09	50 ± 10	3.5 (3.3, 3.7)	NM
CHO/mMT/1	3.8 ± 0.2	32 ± 5	3.6 (3.5, 3.7)	NM
CHO/mMT/2	3.80 ± 0.03	40 ± 4	3.4 (3.4, 3.4)	5.1
CHO/mMT/6	5.2 ± 0.2	87 ± 14	4.7 (4.8, 4.6)	5.7
B16F10[f]	1.00 ± 0.01	6 ± 4	3.8	
B16F10 (Cd-induced)[f,g]	12.3	55 ± 2	11.7	

[a] D_{10}, drug concentration or radiation level required to reduce colony formation in plated cells to 10%.
[b] ND, not detected.
[c] NM, not measured.
[d] Treated with 37 μM $ZnCl_2$ or 10 μM $CdCl_2$ for 24 hr before MT measurement and cisplatin or radiation treatment.
[e] The average of two D_{10} values derived from separate experiments is given. Individual values are shown in parentheses.
[f] Data taken from Koropatnick and Pearson (22).
[g] Treated with 10 μM $CdCl_2$ for 24 hr before MT measurement and cisplatin treatment.

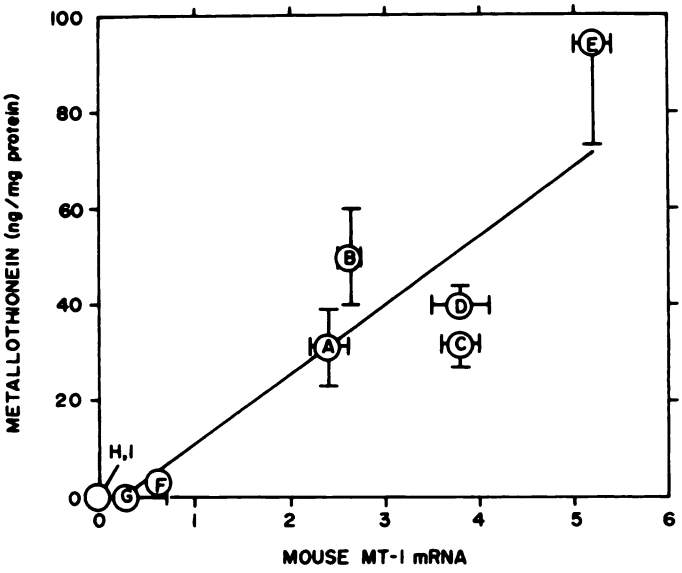

Fig. 3. Correlation between mMT-1 mRNA and protein expression in CHO cells. *A*, CHO/mMT/3; *B*, CHO/mMT/5; *C*, CHO/mMT/1; *D*, CHO/mMT/2; *E*, CHO/mMT/6; *F*, CHO/*neo*/1; *G*, CHO/*neo*/2; *H*, CHO/P/1; *I*, CHO/P/2. Values are plotted as mean ± standard error. Where no *error bars* appear, the standard error was less than the size of the *symbol*.

neo, and CHO/mMT clonal populations were tested for ability to form colonies in the presence of the chemotherapeutic drug cisplatin. All CHO/mMT cells were equally resistant or less resistant to the toxic effects of cisplatin, compared with CHO/P and CHO/*neo* cells (Figs. 4 and 5; Table 1) ($p < 0.01$, by Wilcoxon-Mann-Whitney test for independent samples, indicating that decreased resistance in CHO/mMT cells was significant). CHO/P/1 cells exposed to 37 μM $ZnCl_2$ for 4 hr before treatment with cisplatin had increased cisplatin resistance (Table 1). Cisplatin treatment (1–5 μM, 6 or 24 hr) did not induce

TABLE 2

Cadmium resistance of chimeric cells

The *r* value for MT level versus cadmium resistance was 0.87.

	MT	D_{10}, cadmium
	ng/mg of protein	mM
Control cells		
CHO/P/1	ND[a]	0.8
CHO/P/1 (2-hr Cd)[b]	1.8 ± 0.6	1.2
CHO/*neo*/1	3.3 ± 0.8	1.1
CHO/*neo*/2	2.8 ± 0.7	0.8
CHO/*neo*/1 (2-hr Cd)[b]	3.7 ± 0.3	1.0
mMT-expressing cells		
CHO/mMT/3	31 ± 8	2.9
CHO/mMT/5	50 ± 10	2.7
CHO/mMT/1	32 ± 5	3.3
CHO/mMT/2	40 ± 4	1.9
CHO/mMT/6	87 ± 14	4.2

[a] ND, not detected.
[b] Cells were treated with 0.5 mM $CdCl_2$ for 2 hr and D_{10} values were measured as described in Table 1 and the legend to Fig. 4.

MT mRNA or protein accumulation (Ref. 22 and data not shown). When all cells (controls as well as mMT-expressing cells) were considered together, there was no correlation between cisplatin resistance and MT mRNA ($r = -0.29$) or protein ($r = -0.11$) accumulation. On the other hand, the degree of MT expression, at both the mRNA and protein levels, correlated positively with cisplatin resistance among the five transfectants expressing mMT-1 (Fig. 5; Table 1); CHO/mMT/6, which expressed 2.2 times as much MT-1 mRNA and 2.8 times as much MT protein as did CHO/mMT/3, was 2-fold more resistant to inhibition of colony formation by cisplatin and equal in cisplatin resistance with CHO/P and CHO/*neo* cells. Among CHO/mMT cells, the correlation coefficient for an association with cisplatin resistance was 0.88 for MT protein and 0.84 for MT-1 mRNA. There was no difference in resist-

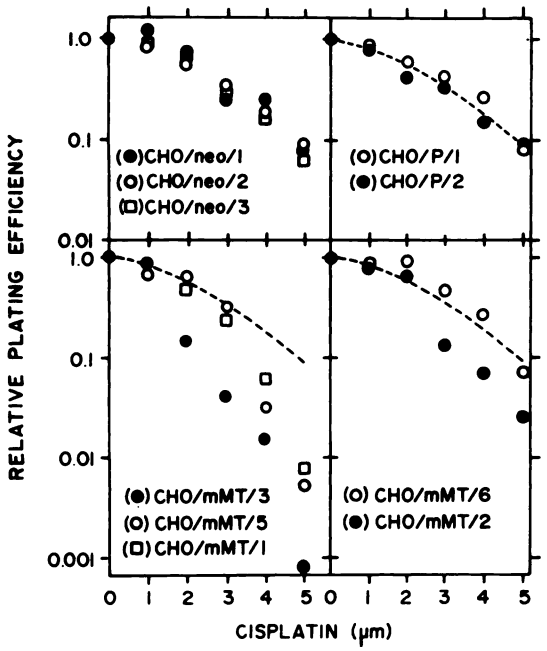

Fig. 4. Colony-forming ability of cells treated with cisplatin as described in Materials and Methods. – – –, D_{10} versus [cisplatin] for CHO/*neo*/3 cells (control cells transfected with pSV2*neo* plus pBR322 only). Standard error in relative plating efficiency was less than the size of the symbols.

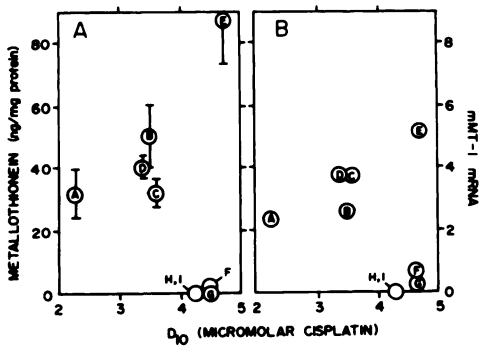

Fig. 5. Correlation between cellular sensitivity to cisplatin toxicity (D_{10}) and mMT-1 protein accumulation (A) and MT mRNA accumulation (B) in CHO cell populations. *Letters within symbols*, cell lines as defined in the legend to Fig. 3. D_{10} values are the average of two independent determinations (from Table 1). MT mRNA and protein values are plotted as mean ± standard error; where no *error bars* appear, the standard error was less than the size of the *symbol*.

ance to ionizing radiation between mMT cDNA transfectants and control cells (either CHO/P or CHO/*neo*) (Table 1).

Growth and survival in the absence of cisplatin. Because a homeostatic role for MT in the regulation of essential metals has been postulated (1, 2, 29), inappropriate mMT expression in CHO cells could have decreased the ability of those cells to form colonies. If so, CHO/mMT cells would exhibit decreased plating efficiency, in comparison with CHO/*neo* controls. The negative association between plating efficiency in the absence of drug and MT mRNA and protein expression is shown in Fig. 6; the correlation coefficient for MT mRNA and plating efficiency was −0.99 and that for MT protein and plating efficiency was −0.95. On the other hand, the growth rate of cells expressing mMT was not significantly different from that of control cells, nor did G418 exposure affect the growth rate of G418-resistant cells (Table 3). There appears, therefore, to be more than one effect associated with expression of mMT in CHO cells. First, expression of transfected mMT-1 genes had a deleterious effect on the ability of CHO cells to form colonies in nutrient medium, without affecting growth rate. Second, mMT-1 expression decreased cellular resistance to cisplatin in a way that cannot be simply correlated with MT accumulation level. Third, mMT-1 expression increased cellular resistance to cadmium, with a good correlation between MT level and the degree of increased resistance.

Discussion

There have been several reports of transformation studies using a BPV vector expressing human MT-II_A (9–11, 13, 14) that mediated expression of high nonphysiological levels of MT. They have shown that there is, associated with viral promoter-mediated expression (and BPV transformation), increased cellular resistance to toxic agents. We transfected mMT-1 cDNA sequences into CHO cells to observe the effect of constitutive MT expression on cellular drug resistance without heavy metal induction or viral transformation. Because the pBR322 vector harboring mMT-1 cDNA has no eukaryotic promoter sequences, mMT-1 expression relied on the action of transcriptional control elements in the CHO DNA in which the mMT-1 cDNA resided. mMT-1 cDNA was incorporated into high molecular weight DNA in the five colonies selected on the basis of MT mRNA expression (Fig. 1) and, presumably, in the majority of 200 colonies chosen on the basis of G418 resistance (three of four nonexpressing colonies contained mMT-1 cDNA). However, it was expressed in relatively few cases (five of 200 G418-resistant colonies, or 2.5%) in the absence of heavy metal induction (Fig. 2; Table 1). All five MT-expressing CHO/mMT/*neo* cell populations accumulated MT protein to levels as high, or higher, than those in CHO/P cells induced with heavy metals (Table 1). Two of the four control CHO/*neo*/*n* cell lines tested expressed some MT, in comparison with undetectable MT levels in CHO/P/*n* cells. It is not known whether this was due to exposure to G418 or expression of CHO MT genes due to transient expression conditions (perhaps increased cellular zinc levels) (30). Nevertheless, MT expression in CHO/mMT/*n* cells was at least 10-fold higher than that in CHO/*neo*/*n* cells and ranged from 31 to 87 ng of MT/mg of protein (the highest level was >2-fold higher than that observed in CHO/P cells maximally induced with cadmium) (Table 1).

Constitutive mMT expression in CHO cells at physiological levels had a deleterious effect on the ability of the cells to form colonies on tissue culture plates. The negative relationship with MT protein expression ($r = -0.95$) or MT mRNA accumulation ($r = -0.83$) (Fig. 6) suggested that mMT-1 expression in CHO cells and/or MT expression through heterologous promoters disrupted homeostatic processes involved in survival. The disruption, however, did not affect growth rate or resistance to ionizing radiation; there were no significant differences in these respects between control cells and those expressing mMT. In addition, there was no correlation ($r = -0.43$) between mMT expression and growth rate (Table 3). It is a formal possibility that incorporation of mMT-1 cDNA contributed to decreased CHO colony formation and cisplatin sensitivity by disrupting host genes. However, the variable restriction pattern of incorporated mMT-1 sequences (Fig. 1) suggested different integration sites among the five cell lines tested; disruption of an important host gene in each case seemed unlikely. Most impor-

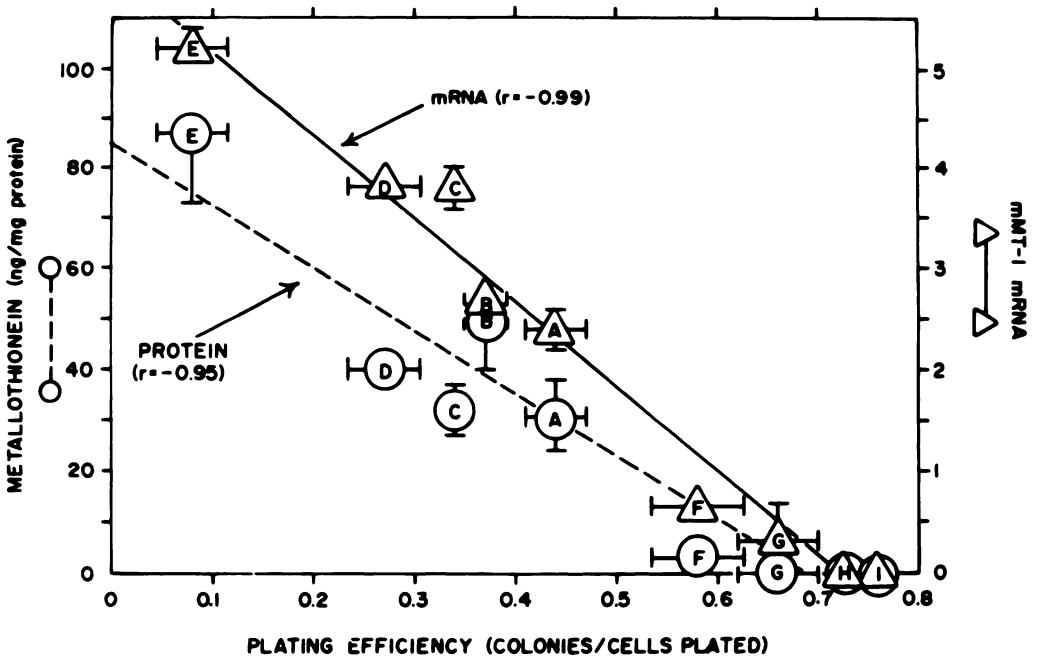

Fig. 6. Negative correlation between plating efficiency and mMT-1 mRNA accumulation (△) and MT protein accumulation (○) in CHO cell populations. Values for mMT-1 mRNA are relative to β-actin mRNA accumulation and are dimensionless. *Letters within symbols*, cell lines as defined in the legend to Fig. 3. Values are plotted as mean ± standard error; where no *error bars* appear, the standard error was less than the size of the *symbol*.

TABLE 3

Growth rate of chimeric cells

All cells were grown in 400 μg/ml G418 sulfate (except where indicated), and doubling time was assessed as described in the text.

Cells	Doubling time
	hr
CHO/P/1 (no G418)	23
CHO/*neo*/1 (no G418)	27
CHO/*neo*/1	29
CHO/*neo*/3	24
CHO/mMT/3	27
CHO/mMT/2	31
CHO/mMT/6	24

tantly, CHO cells expressing mMT had elevated resistance to cadmium toxicity that correlated well with the level of MT expression (Table 2). Therefore, MT expressed from transfected mouse cDNA was functional, and incorporated sequences did not impair constitutive genes such that the ability of cells to exhibit a resistant phenotype was precluded. Moreover, two clones transfected with control vectors alone had no significant decrease in plating efficiency, and decreased plating efficiency correlated well with transfected MT gene expression overall (Fig. 6). These data strongly suggest a role for transfected MT expression (and not disruption of host genes) in decreased CHO cell survival. Although such a homeostatic role for MT is suggested by its multiple DNA regulatory elements, developmentally regulated expression, and ability to bind physiologically important zinc and copper molecules, the observation of specifically altered MT expression leading to decreased cell survival (perhaps due to functional differences between transfected mouse and host CHO MT genes) offers the first direct evidence of a such a homeostatic role for MT.

All five clonal populations of CHO cells expressing mMT were equally resistant or less resistant, compared with controls (i.e., CHO/*neo*/*n* and CHO/P/*n* cells). Thus, mMT production in the CHO cells had the general effect of decreasing cisplatin resistance. On the other hand, when CHO/mMT/*n* clonal populations were compared among themselves, without reference to cells that did not express mMT, there was a good correlation between mMT level and cisplatin resistance (r = 0.84) (see Fig. 5), indicating that mMT expression could enhance cisplatin resistance. These observations are not contradictory if MT plays multiple roles in cells. MT may indeed act as a protective scavenger of reactive electrophiles, as suggested by others (10, 13, 14), to produce a protective effect among mMT-expressing CHO cells. However, the generally deleterious effect on drug resistance suggests that expression of foreign mMT in a CHO context disrupted MT functions distinct from radical scavenging. These results suggest that MT plays more than one role in cells and is important in processes (a) fundamental to cell survival and/or maintenance and (b) contributing directly to cellular drug resistance.

When cells were tested for resistance to ionizing radiation, foreign MT expression appeared to have no effect, at least among the limited number of cell lines tested (Table 1). This is consistent with results obtained by others (10, 11) and indicated (along with the observed MT-mediated increase in cadmium resistance) that the cellular processes affected by perturbation of MT in CHO cells were not so general that they affected cellular responses to toxic events of all types.

The markedly decreased colony-forming ability of chimeric mMT-producing CHO cells in the absence of cisplatin (Fig. 6) could not account for the general decrease in D_{10} values for all five CHO/mMT populations. Cell survival in each case was measured as a proportion of survival in the absence of drug; the effect of variable plating efficiency among transfectants was, therefore, corrected for. One possibility to account for enhanced cisplatin toxicity in CHO/mMT/*n* cells is that foreign MT expression (under the control of heterologous promoters and in the absence of a coordinated cellular response to

stress involving multiple genetic loci) enhanced cisplatin toxicity in a fashion that was not evident in cells expressing their own MT as part of a multigene response to heavy metal induction (8–10, 22). In fact, CHO cells expressing CHO MT in response to zinc induction had increased resistance to cisplatin (Table 1), similar to zinc-treated mouse melanoma cells (22).

The increased cisplatin resistance reported under some circumstances (10) is not clearly reconcilable with the generally decreased cisplatin resistance in CHO cells expressing mMT reported here. However, differences in methodology could have affected the nature of the perturbation of drug resistance; in contrast to the report of Kelley *et al.* (10), we tested cells that were not transformed by BPV and expressed lower, more physiological, levels of MT. Furthermore, our cells were not selected for overexpression of MT by growth in medium containing heavy metals (cadmium or zinc), a treatment that induces or selects for multiple characteristics that might mediate cisplatin resistance (13, 22) (see results for zinc-treated CHO/P/1 cells in Table 1). In addition, the consequences of expressing mMT, rather than human or CHO MT, in CHO cells are not clear in the absence of information about the physiological function of MT. Nevertheless, it is obvious that perturbed MT expression in CHO cells is reflected in altered drug resistance. The decrease in cisplatin resistance in cells transfected with active MT genes may be a consequence of disrupted homeostatic processes in which MT has a critical role.

In summary, we showed that CHO cells expressing mMT-1 had decreased ability to form colonies in the absence of toxic agents, decreased resistance to cisplatin toxicity, and increased resistance to cadmium. This suggests that multiple cellular processes (including both homeostatic functions and processes important in drug resistance) were altered in host CHO cells. These data suggest that CHO cells respond to the toxic effects of cadmium and cisplatin through pathways involving MT; however, the role of MT may be different in response to each toxin. The deleterious effect on cell viability provides evidence for the postulated role of MT in normal cellular growth and maintenance. The concomitant alteration in MT expression and cisplatin resistance among CHO cells expressing foreign MT genes suggests that MT may play an important role in cellular drug resistance.

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