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Overexpression of Metallothionein Confers Resistance to Anticancer Drugs

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Resistance to antineoplastic agents is the major obstacle to curative therapy of cancer. Tumor cell lines with acquired resistance to the antineoplastic agent *cis*-diamminedichloroplatinum(II) overexpressed metallothionein and demonstrated cross-resistance to alkylating agents such as chlorambucil and melphalan. Human carcinoma cells that maintained high levels of metallothionein because of chronic exposure to heavy metals were resistant to *cis*-diamminedichloroplatinum(II), melphalan, and chlorambucil. Furthermore, cells transfected with bovine papilloma virus expression vectors containing DNA encoding human metallothionein-II_A were resistant to *cis*-diamminedichloroplatinum(II), melphalan, and chlorambucil but not to 5-fluorouracil or vincristine. Thus, overexpression of metallothionein represents one mechanism of resistance to a subset of clinically important anticancer drugs.

CIS-DIAMMINEDICHLOROPLATINUM (CP) is one of the most widely used antitumor agents, forming the cornerstone of chemotherapy of testicular, ovarian, and small cell lung carcinomas. As an electrophilic metal coordination complex, CP interacts with DNA to form bidentate adducts (*1*), thus exerting an effect similar to other therapeutically valuable antitumor alkylating agents, such as melphalan and chlorambucil. The development of resistance to antineoplastic agents significantly restricts their efficacy (*2*). Therefore, identification of the mechanisms of antineoplastic drug resistance is a critical precursor to the design of new treatment strategies for cancer. The development of resistance to CP or the alkylating agents has been explained by several factors, including reduced drug internalization, increased DNA repair processes, and an increase in inactivating proteins such as cellular thiols (*1, 3, 4*).

Metallothioneins (MTs) are cysteine-rich proteins that are present in a wide variety of eukaryotes and that constitute the major fraction of the intracellular protein thiols. They complex with group II_B metals and function extensively in Zn^{2+} and Cu^{2+} homeostasis and heavy metal detoxification (*5*). In addition, indirect evidence shows that the abundant nucleophilic sulfhydryl groups in MT can interact with many electrophilic toxins, participate in controlling intracellular redox potential, and act as scavengers of oxygen radicals generated during the metabolism of xenobiotics (*5*). The transcription of the MT gene family is controlled both by metals and by nonmetal environmental stimuli such as epinephrine, glucocorticoids, thermal injury, cytokines, cyclic nucleotides, and phorbol esters (*5*).

Metallothioneins are attractive candidates as modulators of cellular sensitivity to electrophilic anticancer agents (*6–10*), although no direct evidence to support their involvement has yet been reported. Several investigators (*9, 10*) have observed an increase in

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Table 1. Relation between MT content and drug resistance. The cell lines used were SCC-25, human head and neck carcinoma; G3361, human melanoma; SW2, human small cell carcinoma; SL6, human large cell carcinoma; A-253, human head and neck carcinoma; and L1210, murine lymphocytic leukemia. The drugs used were CP; Cd, $CdCl_2$; Blm, bleomycin; and DACH. Resistance ratio is defined as the ratio of CP concentration required to inhibit cellular proliferation by 50% (IC_{50}) in resistant cells to the IC_{50} of the parental cells. Cells in exponential growth were treated with various concentrations of CP; IC_{50} values of human tumor cell lines were determined as described (*9, 16*). We determined the IC_{50} for murine L1210 cells (in suspension) by counting the number of cells after they were treated with continuous exposure to various concentrations of CP for 3 days (*17*).

Cell line	CP resistance ratio	MT content* (fold increase ± SEM)
SCC-25/CP	7.1	4.37 ± 0.50
G3361/CP	6.7	2.00 ± 0.10
SW2/CP	4.5	5.10 ± 0.50
SL6/CP	2.5	3.37 ± 0.44
MCF-7/CP	2.5	0.95 ± 0.11
A-253/Cd	2.7	3.27 ± 0.37
A-253/C-10/Blm	0.88	0.72 ± 0.04
L1210/CP	44	13.3 ± 1.50
L1210/CP-R	5.8	2.18 ± 0.15
L1210/DACH	2.7	2.94 ± 0.18

*The MT content was estimated by an indirect competitive ELISA. Polyclonal rabbit antibody to rodent and human MT-I and MT-II showed reactivity at 1:3000 (*12*). Rabbit MT-I and MT-II (50 ng/ml) were fixed to microtiter plates overnight at 4°C. Unbound sites were blocked with phosphate-buffered saline (PBS) containing 1% bovine serum albumin. Cytosolic extracts from tumor cells or known amounts of competitor MT as standards were incubated with the rabbit antiserum and then applied to the microtiter wells. After 2 to 6 hours at 37°C and washing with PBS–Tween 20 (0.05%), a goat antibody to rabbit immunoglobulin G complexed with horseradish peroxidase (HyClone) was added at 1:3000. After incubation at 23°C for 1 hour and three washings, *o*-phenylenediamine (0.4 mg/ml) containing 0.1% urea peroxide in 0.1*M* citrate buffer, *p*H 4.5, was added as substrate. Color development was monitored by ELISA reader at 492 nm. Standard curves were generated with logit methodology (*18*). MT content (nanograms of MT per microgram of protein) (mean ± SE) of the parental lines were SCC-25, 0.46 ± 0.03; G3361, 0.39 ± 0.027; SW2, 0.052 ± 0.002; SL6, 0.115 ± 0.029; MCF-7, 0.09 ± 0.004; A-253, 1.3 ± 0.6; and L1210, 0.46 ± 0.038. Protein content of cytosolic extract was determined by the method of Bradford (*19*). The fold increase in MT was calculated as the ratio of MT in resistant cell lines to that in parental cell lines. Results were from three or more experiments.

total protein sulfhydryls, but not in nonprotein thiols, in tumor cells with acquired resistance to CP. Intracellular binding of CP to MT or MT-like proteins has also been demonstrated in cells that contained high levels of MT (*6, 8*). Although normal and malignant cells with heavy metal–induced MT display cross-resistance to CP and to alkylating agents (*6, 10*), the contribution of MT to the drug-resistant phenotype after heavy metal exposure is uncertain because heavy metals are toxic and can induce various other bioactive substances including stress proteins (*11*). Furthermore, overexpression of MT in CP-resistant cells has not been demonstrated (*10*). Thus, the involvement of MT in conferring resistance to anticancer agents remains controversial. We have now defined a role for MT in determining cellular responsiveness to agents that covalently interact with nucleophilic sites on DNA.

We generated resistant cell lines by repeated exposure to escalating doses of a particular drug (Table 1). The MT content of sensitive and resistant cell lines was determined by enzyme-linked immunosorbent assay (ELISA) with rabbit antiserum to MT (*12*). With the exception of a mammary carcinoma cell line (MCF-7/CP), all the human tumor sublines we examined, including human head and neck carcinoma (SCC-25/CP), small cell carcinoma (SW2/CP), large cell carcinoma (SL6/CP), and melanoma (G3361/CP), showed increased MT compared to levels in the corresponding parental lines (Table 1). Conversely, human head and neck carcinoma cell line A-253 with acquired resistance to $CdCl_2$ also had threefold increase in MT and showed cross-resistance to CP (Table 1). When a population of the same cells was selected for resistance to another anticancer agent, bleomycin, the MT level of the bleomycin-resistant cell line (C-10) was only 70% of that found in the parental cell line (A-253) (Table 1). Moreover, the C-10 cells were slightly sensitive rather than resistant to CP.

Degree of resistance was also associated with MT content in the murine leukemia cell line L1210. Loss of resistance to CP by a revertant cell line (L1210/CP-R) was associated with concomitant lowering in MT content (Table 1). Metallothionein was only moderately increased (2.9-fold) in L1210 cells with acquired resistance (15-fold) to a CP analog, 1,2-diaminocyclohexane platinum sulfate (DACH) (*13*). These cells also displayed modest cross-resistance to CP, an indication that the mechanism of DACH resistance in these cells might be distinct from that of CP.

Therefore, MT is frequently overproduced in tumor cells with resistance to CP (Table 1). There are, however, several known mechanisms of resistance to anticancer agents, and we speculate that MCF-7/CP cells (Table 1) and cells in which others have failed to detect an increase in MT (*10*) must use other mechanisms of resistance.

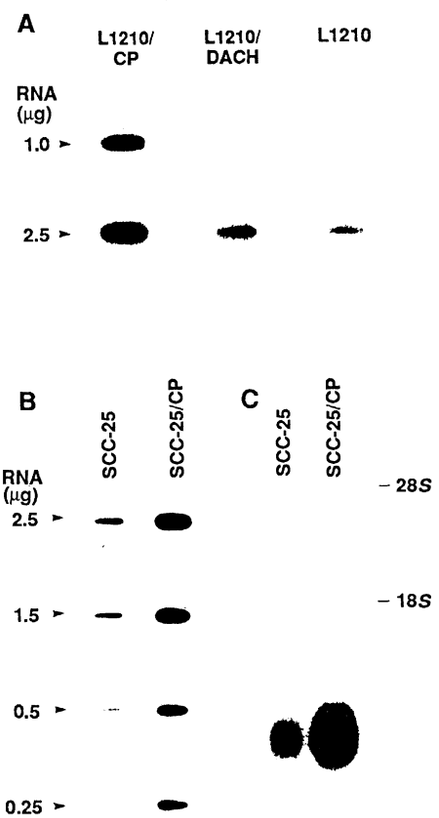

Fig. 1. Quantitation of MT RNA from sensitive and resistant cell lines. (**A**) Dot blot analysis of L1210, L1210/CP, and L1210/DACH cells. (**B**) Dot blot analysis of SCC-25 and SCC-25/CP cells. (**C**) Northern analysis of SCC-25 and SCC-25/CP cells. Total RNA was extracted and purified from 10^7 cells by the guanidinium isothiocyanate–CsCl method (*14*). For dot blot analysis, serial dilutions of denatured RNA were applied to nitrocellulose membrane with a "slot-blot" apparatus. Hybridization was carried out with a radiolabeled 3.0-kb Hind III fragment of human genomic DNA containing MT-II_A (*20*) at 42°C in the presence of 50% formamide as described (*14*). Washing after hybridization was performed in 0.5× saline sodium citrate plus 0.1% SDS at 55°C.

Table 2. Sensitivity of C127 cells transfected with hMT-II_A gene-containing vector to various anticancer agents. Mouse C127 cells transfected with either BPV vector (control) or BPV that contained hMT-II_A gene were treated with various anticancer drugs. Survival curves for each drug were obtained as described in Fig. 2. The data are expressed as IC_{50} (±SE) values calculated from the survival curves; the resistance ratio is the IC_{50} of BPV–hMT-II_A–transfected cells divided by the IC_{50} of BPV-transfected cells. Results were obtained from three to five experiments.

Drugs	IC_{50} (μ*M*)		Resistance ratio
	BPV	hMT-II_A	
CP	1.45 ± 0.26	6.36 ± 1.03	4.43
Melphalan	5.42 ± 0.24	23.4 ± 6.46	4.30
Chlorambucil	18.5 ± 0.76	78.0 ± 6.9	4.22
Doxorubicin	0.265 ± 0.025	0.450 ± 0.07	1.70
Bleomycin	2.67 ± 0.83	4.47 ± 2.00	1.67
5-Fluorouracil	1.00 ± 0.09	1.30 ± 0.21	1.30
Vincristine	0.013 ± 0.001	0.015 ± 0.005	1.15

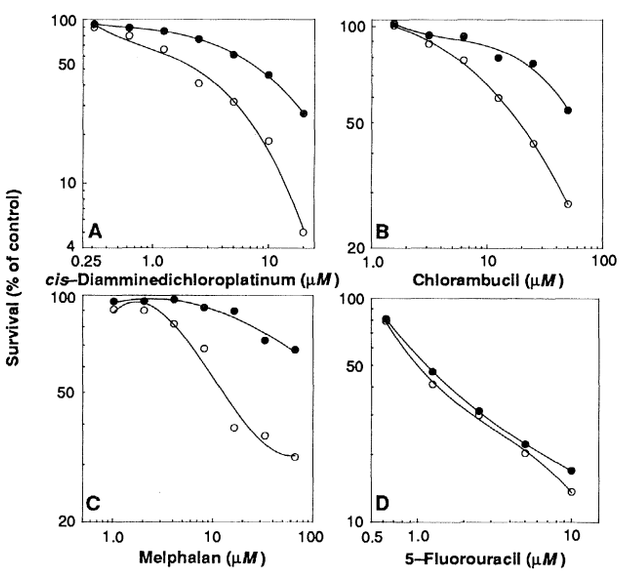

Fig. 2. Survival curves of C127 cells transfected with BPV alone (○) or BPV containing a hMT-II_A gene construct (●). Exponentially growing cells were added to microtiter plates and incubated in the absence or presence of various doses of (**A**) *cis*-diamminedichloroplatinum, (**B**) chlorambucil, (**C**) melphalan, and (**D**) 5-fluorouracil. After 72 hours of incubation, 0.1 mg of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) (Sigma) was added to each well and incubated for 3 hours at 37°C. MTT was reduced to a blue formazan product by living cells. Color development was monitored by an ELISA reader at a wavelength of 540 nm (*16*). These are representative results from three or more experiments.

Analysis of RNA from sensitive and resistant cell lines further supported our conclusion that MT gene expression is increased in drug-resistant cells. Total RNA probed with a 3.0-kb Hind III fragment of human genomic DNA containing MT-II_A (*14*) revealed an increase of MT mRNA in the L1210/CP (tenfold) (Fig. 1A), SCC-25/CP (sixfold) (Fig. 1, B and C), and A-253/CP (13.5-fold) cells. Neither L1210/DACH (Fig. 1A) nor L1210/CP-R showed any increase in MT mRNA. No evidence for MT gene amplification could be seen in SCC-25/CP or L1210/CP cells by DNA blot analysis, suggesting an enhanced rate of gene transcription or increased mRNA stability as the likely cause of MT overexpression.

Long-term exposure to drugs or heavy metals can have multiple effects on cells (*11*). Therefore, to establish a direct role for MT in the acquisition of resistance to anticancer agents, we transfected mouse C127 cells with a bovine papilloma virus (BPV) shuttle vector containing a human MT-II_A (hMT-II_A) gene construct (*15*). Cells transfected with BPV–hMT-II_A had a greater than tenfold increase in MT compared to cells transfected with BPV alone, as shown by ELISA, and were resistant to CP over a wide range of concentrations (Fig. 2). We observed a 4.4-fold increase in resistance to CP in cells transfected with BPV–hMT-II_A as compared to the cells transfected with the BPV vector alone (Table 2). These cells were equally resistant to melphalan and chlorambucil, displayed only modest resistance to bleomycin and doxorubicin, and showed no significant resistance to 5-fluorouracil and vincristine (Fig. 2 and Table 2). Therefore, MT may confer selective resistance to sulfhydryl-reactive alkylating agents. This possibility is substantiated by our observation that tumor cell lines, such as SCC-25/CP and L1210/CP, with acquired resistance to CP, were also cross-resistant to melphalan and chlorambucil but not to bleomycin.

Our results indicate that increased MT content can make tumor cells resistant to clinically important cancer chemotherapeutic agents such as CP, melphalan, and chlorambucil. We have shown that (i) tumor cells with increased MT content are resistant to both alkylating agents and CP, (ii) cells with acquired resistance to CP frequently have an increase in MT and overexpress MT mRNA, (iii) reversal of the CP-resistance phenotype is accompanied by a decrease in MT content, and (iv) introduction of a eukaryotic expression vector encoding MT into cells confers the drug-resistance phenotype. Although additional mechanisms of resistance to agents such as CP appear to exist (*4, 9*), the role of MT in drug resistance may be extensive. Neoplastic cells may express increased MT owing to the presence of activated Ha-*ras* oncogenes (*15*). Furthermore, various stimuli are capable of inducing cellular MT synthesis. Therapeutic agents such as interferons and steroids, which are commonly used in the treatment of malignant diseases, induce MT (*5*) and may induce transient drug resistance. It remains to be determined whether long-term exposure to CP directly induces MT synthesis or whether the high frequency of overexpression of MT in CP-resistant cells reflects selection. A further understanding of the cause of MT overexpression should facilitate the development of therapeutic approaches to circumvent this mechanism of resistance to antineoplastic drugs.

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Induction of B Cell Unresponsiveness to Noninherited Maternal HLA Antigens During Fetal Life

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Patients who have received many transfusions become highly sensitized and develop antibodies against almost all HLA alloantigens, so that finding a cross-match negative kidney donor is difficult. A survey of those patients showed that 50 percent did not form antibodies against the noninherited maternal HLA antigens. Apart from the obvious clinical implications, the data indicate that a human equivalent of murine neonatal or actively acquired tolerance has now been identified.

ACTIVELY ACQUIRED TOLERANCE IN mice to the antigens of the murine major histocompatibility complex (H-2) is induced by exposure of the animals to allogeneic lymphocytes within 24 hours of birth and often leads to antigen-specific and lifelong immunological nonresponsiveness (*1, 2*). Actively acquired tolerance to the major histocompatibility complex (MHC) in humans (HLA) cannot be studied in the same way. However, we have evidence for the existence of neonatal tolerance in humans in a study of 26 highly sensitized patients waiting for a renal allograft. These patients had developed complement-dependent antibodies to the HLA antigens of almost all unrelated caucasoid donors. It is difficult to find a kidney donor for such patients, as the cross-match with almost all potential donors is positive, ex-

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