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METALLOTHIONEINS AND CELL DEATH BY ANTICANCER DRUGS

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ABSTRACT

Both pharmacologic and genetic methods are now available to manipulate intracellular levels of the protein thiol metallothionein. These approaches have begun to reveal the protective roles metallothioneins (MTs) have against oxygen, nitrogen, and carbon-centered free radicals, as well as the contributory role of MT to resistance to a broad range of electrophilic therapeutic agents, including antineoplastic drugs. We suggest MTs are enlisted to act as primative antioxidant defense mechanisms in mammalian cells and, thus, may have widespread importance in the biology of cell death.

PERSPECTIVE

The extensive presence of toxic electrophiles in the environment mandates that organisms elaborate both intra- and extracellular protective substances. We know from studies with prokaryotes and single-cell eukaryotes that both oxidative injury and DNA damage invoke phenotypic changes, including increased transcription of protective proteins (1). Mammalian cells also respond to oxidative stress and DNA damage with transcriptional activation, but we are just beginning to understand the signaling systems that these cells use to detect and respond to potentially damaging substances. Moreover, the functionality of most of these induced proteins is not certain (2). The electrophilic nature of toxic substances calls attention to the role of intracellular

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nucleophiles as targets of potentially injurious substances. Interestingly, the major intracellular thiol-containing proteins, metallothioneins (MTs), are induced by many substances, including UV radiation, DNA damaging agents, and hormones and cytokines, whose levels are elevated by oxidative stress (2–5).

Three major MT isoforms—MTI, -II, and -III—are expressed in mammals; in humans five type I isoforms have been identified, in addition to MTIIA and -III, each encoded by unique but highly homologous genes (5, 6). Because MTs can bind metals, MT proteins have been of interest to physiologists and nutritionists concerned with zinc homeostasis and to toxicologists fascinated with cadmium (Cd) and heavy metal metabolism and detoxification. Protein chemists, spectroscopists, and crystallographers have analyzed this relatively small molecule to understand the relationships between structure and function. Molecular biologists intrigued with transcriptional control have found the genomic regions that control MT expression to be rich sources of information. Consequently, there are now several excellent reviews on these subjects (5–11). We focus most of our comments on an important but more problematic aspect of MT—namely, its function, and especially its role in protecting cells against death induced by anticancer drugs. We suspect this discussion is of special interest to pharmacologists, toxicologists, and medical oncologists.

METALLOTHIONEINS AND ELECTROPHILIC ANTICANCER DRUG–MEDIATED CELL DEATH

Resistance to anticancer drugs is the single most important factor in the failure of curative conventional cancer chemotherapy. Because antineoplastic agents have both diverse chemical structures and mechanisms of action, the wide variety of putative resistance mechanisms is not surprising. What makes the MTs most interesting is their potential involvement in the resistance to many different pharmacological agents. In this review we examine the conflicting evidence for the role of MTs in anticancer drug resistance by focusing on specific classes of agents. Many clinically used anticancer agents are electrophilic, and their mechanism of action is thought to be related to their ability to generate covalent DNA inter- and intra-strand cross-links. Conners (12) was the first to demonstrate the protective potential of thiols against the toxicity of alkylating agents, and he postulated a role for thiols in anticancer drug resistance. MTs are nucleophilic, with 30% of their amino acids being cysteines, and MT expression increases in some tissues after in vivo treatment with DNA alkylating agents (3). These findings stimulated investigators to examine the potential of MTs to protect cells against electrophilic anticancer drugs and mutagens. Three distinct approaches have been used: cell culture systems,

Table 1 A list of studies examining potential protective actions of MT in cells or organisms treated with electrophilic anticancer drugs or mutagens

Agent	Effect[a]	Species	Model	Reference
Cisplatin	+/−	Hamster	Cell culture	24
Cisplatin	+	Hamster	Cell culture	22
Cisplatin	+	Human	Cell culture	15
Cisplatin	+	Human	Cell culture	19
Cisplatin	+	Human	Cell culture	30
Cisplatin	−	Human	Cell culture	21
Cisplatin	+	Human, murine	Cell culture	13
Cisplatin	+	Human, murine	Cell culture	16
Cisplatin	+	Human, murine	Cell culture	64
Cisplatin	−	Murine	Cell culture	23
Cisplatin	+	Murine	Cell culture	18
Cisplatin	+	Murine	Cell culture	20
Cisplatin	+	Human	Tumors	38
Cisplatin	−	Human	Tumors	39
Cisplatin	+	Human	Tumors	36
Cisplatin	+	Murine	Mice	19
Cisplatin	+	Murine	Mice	44
Cisplatin	+	Murine	Mice	41
Cisplatin	+	Murine	Mice	43
Cisplatin	+	Murine	Mice	45
Cisplatin	+	Murine	Mice	54
Cisplatin	+	Murine	Mice	101
Cisplatin	+	Murine	Rats	46
Cisplatin	−	Murine	Rats	47
Cisplatin	−	Murine	Rats	48
Cisplatin	+	Human	Clinical	50
Cisplatin	+	Human	Clinical	51
Cisplatin	+	Human	Clinical	52
Carboplatin	+	Murine	Mice, rats	48
Chlorambucil	+	Murine	Cell culture	14
Chlorambucil	+	Murine	Cell culture	16
Melphalan	−	Hamster	Cell culture	17
Melphalan	+	Murine	Cell culture	16
Mitomycin C	+	Hamster	Cell culture	25
Mitomycin C	NC	Murine	Mice	54
MNNG[b]	+/−	Hamster	Cell culture	26
MNNG	−	Hamster	Cell culture	27
MNNG	+	Hamster	Cell culture	25

[a] Protection from toxicity is indicated with +; no change, NC; enhancement of toxicity, −.

[b] MNNG, N-methyl-N-nitrosourea.

human tumor samples, and intervention studies with animals and humans (Table 1).

Metallothionein Induction and Gene Transfer Studies in Cell Culture

Two general strategies have been adopted for cell culture systems: (*a*) analysis of cell sensitivity to anticancer drugs after MT levels have been elevated either pharmacologically or genetically and (*b*) analysis of the MT levels in cell lines with acquired or intrinsic resistance to the electrophilic agent of interest. Bakka et al (13) were the first to provide evidence that MTs could modulate the cytotoxicity of any anticancer drug. They used human and murine cell models that had been chronically exposed to Cd. The Cd-resistant cells had a three- to fourfold increase in total sulfhydryl content, and 2–3% of the total protein was MTs compared to only trace amounts of MTs in the parental cells (13, 14). The growth rates, nonprotein thiol levels (i.e. glutathione levels), cell-cycle phase distribution, and DNA-repair capacity of the Cd-resistant cells were indistinguishable from the parental cells. The Cd-resistant cells were approximately two- to threefold cross-resistant to *cis*-diamminedichloroplatinum(II) (cisplatin). Furthermore, these cells were found to be cross-resistant to chlorambucil, another electrophilic anticancer drug (14). Based on other studies (15, 16), Cd-resistant cells that overexpress MT always appear to be cross-resistant to cisplatin and electrophilic anticancer drugs. Interestingly, brief treatment of Chinese hamster ovary (CHO) cells with concentrations of Zn that do not cause an increase in MTs produces resistance to the cytotoxicity of cisplatin and the electrophilic anticancer drug melphalan (17, 18).

Nonmetal inducers of MT also can increase cellular resistance to cisplatin. A 24-h pretreatment of human hormone-independent prostatic tumor cells (DU-145) with interleukin-1β causes a two- to threefold increase in MT protein and a two- to threefold increase in resistance to cisplatin (19). Both increased MT expression and increased cisplatin resistance were blocked by concomitant incubation of cells with a recombinant human interleukin-1 receptor antagonist. Another model that has been used to study the effects of nonmetal MT inducers is a strain of normal rat kidney cells (6m2) infected with a temperature-sensitive *v-mos*-containing Moloney murine sarcoma virus (20). At the permissive temperature (<33°C), the cells express functional *v-mos* and are transformed, whereas at the nonpermissive temperature (37–39°C) these cells fail to express functional *v-mos* and are nontransformed. Expression of *v-mos* prevents nuclear retention of the glucocorticoid receptor and transcriptional activation of MT. Thus, a 24-h pretreatment of the nontransformed cells with 0.5 μM dexamethasone induces significant MT and causes transient resistance to cisplatin; in contrast, similar treatment of the *v-mos*-expressing cells pro-

duces neither an increase in MT expression nor an increase in cisplatin resistance. Thus, in this model dexamethasone protection against cisplatin toxicity was selective for the cells not expressing *v-mos* and occurred in conjunction with a transient increase in MT.

Substances that induce MT, such as metals, cytokines, or dexamethasone, also affect the expression of many other genes, which obfuscates any conclusions. MT genes and their promoters have been well characterized, making gene transfer studies attractive approaches to examine the functional role of MTs. High levels of MT expression have been obtained in mouse cells by using a bovine papilloma virus (BPV)–derived, autonomously replicating vector that contains a human *MTIIA* gene (16). These transfected cells are resistant to cisplatin, chlorambucil, and melphalan but not to functionally unrelated anticancer agents such as 5-fluorouracil or vincristine. This vector can be easily lost because it is not integrated into the host genome. Thus, in our studies (16) infected cells were maintained in low Cd concentrations to ensure the retention of the BPV containing the human *MTIIA* gene. Schilder et al (21) did not see cisplatin resistance with this construct. Although a satisfactory explanation for this failure has not been reached, the autonomously replicating vector may have been unknowingly lost in the absence of metal selection in that study (21), or Cd may have coinduced resistance factors in our study (16). Kaina et al (22) infected CHO cells with a BPV containing the human *MTIIA* gene and reported resistance to alkylating agents, such as mitomycin C and N-methyl-N-nitrosourea, as well as some resistance to cisplatin; they saw no resistance to γ-radiation or the DNA cleaving drug bleomycin. Morton et al (23) found no change in the cisplatin sensitivity of NIH3T3 cells transfected with a plasmid that encodes murine MTI.

Because clonal variation may occur within a population of cells, Koropatnick & Pearson (24) examined five individual CHO clones after transfection with mouse MTI cDNA and noted an excellent correlation between MT levels and the degree of cisplatin resistance. Surprisingly, as a group the transfectant clones were more sensitive to cisplatin than the nontransfected control cells, and this increased sensitivity has not been explained. Furthermore, expression of MT following gene transfection appeared to inhibit the colony-forming ability of CHO cells. Incorporation of the mouse MTI cDNA may have contributed to decreased CHO colony formation and may have altered cisplatin sensitivity by disrupting host genes. In a series of studies using CHO cells, Lohrer and coworkers (25–27) have noted that the sensitivity profile to DNA alkylating agents following MT gene transfer depends on the phenotype of the recipient cells. Transfectants were generated by using BPV containing the human *MTIIA* gene and cells derived from mutant CHO cells lines sensitive to the alkylating agent methanesulfonate. These cell lines were chosen because of their apparent deficiency in DNA-repair enzymes, which led to hypersen-

sitivity to alkylating agents and allowed low levels of drugs to be used. MT gene transfer and MT expression were found to cause resistance, hypersensitivity, or no change in responsiveness to N-methyl-N-nitrosourea depending on which mutant CHO cell line was infected (26, 27).

Acquired and Intrinsic Drug Resistance in Cell Culture

Although increases in total protein sulfhydryls in cultured human tumor cells with acquired resistance to cisplatin implied an increase in MT (15, 28), Kelley et al (16) were the first to demonstrate, both at the protein and mRNA level, that this represents MT overexpression in human and murine cells. Five cisplatin-resistant and -sensitive pairs of human tumor cells were examined; in four pairs of cisplatin-resistant cells MT protein levels were increased between two- and fivefold (16). A murine L1210 leukemia cell line that was 44-fold resistant to cisplatin contained 13-fold more MT than the parental cells. Removal of the selection pressure (cisplatin) led to the emergence of a population of cells with increased cisplatin sensitivity and decreased MT content. More recently, Yang et al (29) examined specific MT isoform expression in three of these pairs of cisplatin-resistant and -sensitive cells. The primary isoform increased in all cells was MTIIA, which generally is the most commonly expressed MT isoform in human cells. MTIIA transcription rate was increased in the cisplatin-resistant cells, and 5-azacytidine treatment only affected the expression of MTIIA in the cisplatin-sensitive cells. Restriction analysis revealed no obvious hypomethylation of the MTIIA regulatory region, suggesting DNA hypomethylation of a *trans*-acting regulatory gene could be responsible for the overexpression of MTIIA in the drug-resistant cells (29). Kasahara et al (30) found elevated MT levels in two human small-cell lung cancer cell lines that were 6- and 11-fold resistant to cisplatin. These cells also were cross-resistant to Cd and electrophilic anticancer agents such as 4-hydroperoxycyclophosphamide and 3-[(4-amino-2-methyl-5-pyrimidinyl)-methyl]-1-(2-chloroethyl)-1-nitrosourea. Mouse fibrosarcoma cells with sevenfold resistance to cisplatin exhibited elevated levels of MTs (31). Although alkylating agents administered in vivo have been shown to increase MTs, cisplatin does not appear to acutely affect MT expression in cultured cells (21). Thus, the in vivo MT induction may be a secondary effect. Moreover, MT overexpression is not a universal phenotype of cisplatin-resistant cells (15, 16, 21, 32).

Tumor Samples

Immunohistochemical studies of human tumor samples provide additional evidence for a role of MTs in resistance to anticancer agents. Cherian and coworkers (33) demonstrated the presence of MTs in 31 of 34 human thyroid archival paraffin-embedded tumor samples. In contrast less than 20% of the

normal thyroid glands were positive for MTs. MTs have now been observed in testicular tumors (34, 35), bladder carcinomas (36), breast carcinomas, and colorectal adenocarcinomas (37). In a small study of eight human transitional cell carcinomas of the bladder, no obvious correlation could be found between the presence or degree of MT staining in the invasive or mucosal portions of the tumor and clinical outcome (36). Normal uroepithelium stained strongly, however, in all three of the patients who had disease progression and died, as opposed to only one of five who were without evidence of disease. MT expression was examined in 19 bladder tumors by Northern analysis, and MT gene expression was found in some tumors that had failed cisplatin chemotherapy (38). In a study of 33 primary testicular germ cell tumor specimens, a distinct difference in the amount of staining was noted between seminomas and nonseminomas (34). Pure seminomas showed little or no MT staining, irrespective of the clinical stage, while nonseminomas, especially those in more advanced clinical stages, stained heavily for MTs. Interestingly seminomas are very sensitive to chemotherapy and radiotherapy. Similarly radio- and chemotherapy-resistant adenomas also showed intense staining, consistent with their potential role in protecting cells against therapeutic agents. Murphy et al (39) investigated the MT levels in 48 ovarian tumors obtained either before or following chemotherapy with alkylating or platinating agents and found more than a 100-fold greater level of MTs in tumors than in normal ovaries. The total MT level did not, however, correlate with response to therapy, age, stage, histology, or tumor cell differentiation state.

The failure to see correlation with these parameters or to find a difference in the level of MTs before and after chemotherapy may reflect the limitation of assaying homogenized tissues. Thus, immunohistochemical methods to assess the level of MT expression in malignant populations seem most likely to reveal a role of MTs in drug resistance.

One surprising aspect of the immunohistochemical studies has been the nuclear and cytoplasmic compartmentalization of MTs. Because MTs have a relatively small molecular mass, most investigators anticipated that they would be uniformly distributed throughout the cell. Nevertheless, some cells have a nuclear phenotype while others have a more cytoplasmic phenotype (36, 40). Neither the functional significance of the distributional differences nor the factors that control it are known, but further investigations on this topic may clarify some of the above-mentioned discrepancies in experimental results.

Animal and Clinical Studies

The first experimental in vivo data to support a protective role of MTs was obtained by Naganuma et al (41), who pretreated mice with a single dose of Cu, Hg, Bi, Cd, Hg, Ni, or Co for 24 h prior to a single cisplatin injection of 35 μmol/kg s.c. They found that metal pretreatment decreased the mortality

of cisplatin compared to vehicle treatment, as quantified by the number of mice surviving 15 days after cisplatin. Treatment of tumor-bearing mice with 200 μmol Zn sulfate/kg (s.c.) for 2 days increased hepatic, renal, and tumor MTs and protected both the kidneys and the tumors from the toxic actions of cisplatin (42). Interestingly, coinjection of propargylglycine, which is a specific inhibitor of the cystatathionine pathway and depletes free cysteine pools and MT synthesis, reduced tumor MT but did not affect renal MT levels. Inclusion of propargylglycine with $ZnSO_4$ allowed for the reduction of cisplatin renal toxicity without reducing antitumor activity. Similarly, Bi subnitrate pretreatment elevated renal MT in mice and protected these mice against the renal toxicity of cisplatin (43, 44). The therapeutic index of cisplatin appeared to increase because there was no abrogation of the antitumor activity of cisplatin. Pretreatment of mice with either $ZnCl_2$ or Bi subnitrate also protects mice from the secondary carcinogenic effects of either cisplatin or melphalan (45).

In contrast to results with mice, the role of MTs in protecting against cisplatin-induced renal toxicity in rats is more controversial. Pogash et al (46) examined the protective action of Zn acetate pretreatment on pituitary, Leydig, and Sertoli cell dysfunction following cisplatin administration to rats. Zn acetate pretreatment (6mg/kg per day for 5 days) partially prevented cisplatin-induced reduction in testicular androgen-binding protein, suggesting a near normalization of Sertoli cell function. In contrast, cisplatin-induced damage to Leydig and pituitary cells was not lessened by Zn pretreatment. Other groups have examined the effect of Zn pretreatment on cisplatin toxicity in rats. Rats fed Zn-enriched diets (5 to 20 mg/kg) have a twofold increase in renal and hepatic MTs but are not protected against the acute renal toxicity of cisplatin, as measured by increased serum urea nitrogen (47). Similar results were seen with rats injected with Zn salts and subsequently examined for cisplatin-induced renal damage, using enzyme excretion and histology (48). A high-Zn diet has, however, been shown to protect rats from the hematotoxicity of carboplatin, a cisplatin analogue, without impairing its antitumor activity against experimental brain tumors (49). Northern blot analysis confirmed that a high-Zn diet induced MT mRNA in rat and mouse kidneys and bone marrow but not in brain tumors. These results suggest that increasing dietary intake of Zn, which does not effectively cross the blood brain barrier, might enhance the therapeutic index of carboplatin in the treatment of brain tumors.

Several clinical trials with Bi salts have been initiated in an attempt to increase the therapeutic index of cisplatin. In a limited clinical trial, ten patients with ovarian cancer were treated with a combination of cisplatin, doxorubicin, and cyclophosphamide with or without pretreatment with oral Bi subsalicylate (1 g, 5 times over 30 h preceding the start of chemotherapy). A small protective effect of Bi treatment on renal function was noted, although its clinical significance is unknown (50). Kondo et al (51) compared the renal status of 29

patients with urogenital tumors treated with combination chemotherapy containing cisplatin. They noted a reduction in the renal toxicity associated with cisplatin administration when patients were pretreated with 1 g of Bi subnitrate 3 times a day and 670 mg Na citrate 3 times a day for 5 days. Saijo et al (52) treated 17 patients with 25 mg of Bi subnitrate/kg for five days before cisplatin treatment and 18 patients without Bi subnitrate. Urinary β_2-microglobulin and N-acetyl-D-glucosaminidase were significantly lower in the Bi-pretreated group, although there were no differences in acute or late emesis or in the degree of granulocytopenia and thrombocytopenia. Although these limited studies suggest Bi pretreatment may reduce cisplatin nephrotoxicity in some patients, it is unclear if such therapy adversely affects the antitumor activity of cisplatin.

METALLOTHIONEINS AND OTHER ANTINEOPLASTIC AGENTS

In addition to the potential protective role of MTs against electrophilic anticancer drugs, there is evidence that MTs can protect cells and animals against the toxicity of other agents used to treat cancer. As indicated in Table 2, protection against topoisomerase II inhibitors, antimetabolites, and DNA-cleaving agents has been reported. In general many of the same approaches used with electrophilic agents have been applied, although the investigations are less extensive. Cd pretreatment of cultured human prostatic DU-145 cells produced a modest resistance to doxorubicin (53). Cells that overexpressed MTs owing to gene transfer also appeared to demonstrate a slight resistance to doxorubicin (16). High doses of doxorubicin are cardiotoxic, and pretreatment of mice with Bi subnitrate affords cardioprotection against doxorubicin (45, 54). A significant relationship between MT expression measured by immunohistochemistry and doxorubicin resistance was seen in 94 human non-small-cell lung carcinomas from previously untreated patients (55). Interestingly, however, a good correlation also exists between MT content and p-glycoprotein or glutathione-S-transferase-π expression (55).

Protection against the toxicity of the antimetabolite 5-fluorouracil has been reported in mice pretreated with Bi (54), although no protection was seen with murine cells expressing high levels of human MTIIA after gene transfer (16). Both in vivo and cell culture studies suggest that expression of MTs does not afford protection against the tubulin binder vinblastine (16, 54). Bakka et al (56) were the first to suggest a protective effect of MT against ionizing radiation when they demonstrated radioresistance in cells with Cd resistance and high MT levels. These same human and murine Cd-resistant cells were later found to be resistant to tumor necrosis factor (57). Cd pretreatment (3 mg/kg, s.c.) of mice increases hepatic MT eightfold and decreases the toxic

Table 2 A list of studies examining the potential protective role of MT in cells or organisms treated with nonelectrophilic antineoplastic agents or radiation

Agent	Effect[a]	Species	Model	Reference
Topoisomerase inhibitors				
Doxorubicin	+	Human	Cell culture	53
Doxorubicin	+	Human	Tumors	54
Doxorubicin	+	Murine	Cell culture	16
Doxorubicin	+	Murine	Mice	42
Doxorubicin	+	Murine	Mice	99
Antimetabolites				
5-Fluorouracil	NC	Murine	Cell culture	16
5-Fluorouracil	+	Murine	Mice	54
Tubulin binders				
Vinblastine	NC	Murine	Cell culture	16
Vinblastine	NC	Murine	Cell culture	54
DNA cleaving agents				
Bleomycin	NC	Hamster	Cell culture	25
Peplomycin	+	Murine	Mice	54
x-irradiation	+	Murine	Mice	100
x-irradiation	+	Murine	Mice	58
x-irradiation	NC	Hamster	Cell culture	25
x-irradiation	+	Murine	Cell culture	56
x-irradiation	−	Murine	Cell culture	59
γ-irradiation	NC	Hamster	Cell culture	22
γ-irradiation	+	Murine	Mice	102
Miscellaneous				
Prednimustine	+	Murine	Cell culture	103
TNF-α	+	Human	Cell culture	57

[a]Protection from toxicity is indicated with +; no change, NC; enhancement of toxicity,

effects of x-irradiation, as measured by decreased mortality rate, increased mean survival time, reduced weight loss, and reduced leukopenia (58). CHO cells that express human MTIIA as a result of gene transfer were not protected against x-irradiation toxicity despite clear resistance to Cd (25). A similar conclusion was reached with murine squamous cell carcinomas with different intrinsic resistance profiles to x-irradiation (59) or with cells exposed to the radiomimetic bleomycin (22, 25). Thus, MTs may have some role in protecting cells against nonelectrophilic anticancer agents, but the protection may be cell-type specific.

MODELS AND GENERAL DISCUSSION

By what mechanism could MT expression provide broad protection against cellular death caused by anticancer drugs, mutagens, and ionizing irradiation?

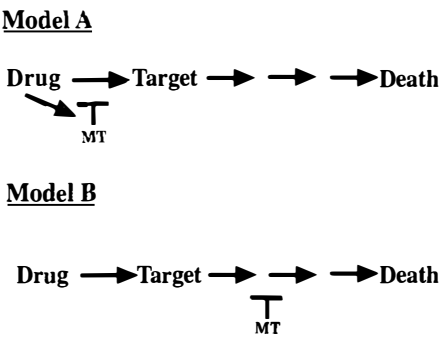

Figure 1 Two models by which MTs could protect cells and tissue from drug-induced death. Inhibition is represented by the T symbol.

Early studies focused on the nucleophilic nature of apothionein and the electrophilic nature of anticancer drugs, such as cisplatin or chlorambucil. A simple schematic of this mechanism is found in Model A of Figure 1. Thus, 24 h after incubating Cd-resistant cells with 15 μM cisplatin, 70% of intracellular Pt was associated with MTs (13). When the Cd-resistant cells were incubated with [^{14}C]chlorambucil, approximately 20 to 40% of the total radioactivity was recovered as radioactively labeled MT, suggestive of intracellular sequestration of chlorambucil or its toxic metabolites (14). In vivo binding of Pt to MT has been seen with rat tissues following cisplatin injections (60). Preinjection of $CdCl_2$ significantly enhanced the amount of Pt associated with MT (60). Incubation of MT-containing rat tissue extracts with cisplatin also produced Pt covalently bound to MT, although 72 h was required to reach equilibrium. Kraker et al (61) found about 20 to 25% of the Pt in Ehrlich cells incubated with cisplatin for 25 h was associated with MTs. Moreover, they noted that cisplatin displaced about 40% of the normal Zn from intracellular MT. Based on in vitro studies with cisplatin and MT, Pt binds stoichiometrically to protein sulfhydryls and can displace Zn (62). During the process of MT binding with Pt, the amine ligands of the cisplatins are lost, suggesting a tetrathiolate coordination for Pt(II) with MTs. The kinetics of the reaction are biphasic. These data appear to support the notion that MT could act to intercept or scavenge electrophilic agents and act as a disposable alternate target.

In contrast to the results described above, several studies argue against Model A in Figure 1. Mason et al (63), for example, found Pt eluted with a low molecular weight protein fraction from cisplatin-treated rat kidneys that had chromatographic properties different from either endogenous Cu, Zn, or Cd MT. Furthermore, they found that pretreatment of rats with Cd had no effect on the uptake or subcellular distribution of Pt in the liver or kidney after

cisplatin treatment and concluded that MT probably did not have a significant role in the renal metabolism of Pt following the administration of cisplatin to rats. Other studies with rats also are inconsistent with the notion that protection against cisplatin nephrotoxicity is due solely to the metal-binding properties of MT (47,48). Montine & Borch (64) found that induction of MT by Cd did not protect quiescent porcine kidney epithelial cells from the toxicity of cisplatin. A number of pharmacological and gene transfer studies point to a protective mechanism that is unrelated to a direct electrophilic scavenging property of MT (18, 24, 26, 27). Boogaard et al (65) suggested that the nephrotoxicity of cisplatin may be produced by drug-induced peroxidation and that MT may have protective antioxidant properties. Nonetheless, the role of oxidative damage in the toxicity of electrophilic anticancer agents is not well established. Recently, however, attention has been directed to oxidative damage as a mediator in drug-induced cell death, possibly as a signal in apoptosis or programmed cell death (66). Specific genes, such as *bcl-2*, prevent cell death and also may disrupt oxidative damage (67). Model B in Figure 1, in which MT acts distal to the drug-target interactions, is an alternative that could not only reconcile disparate data concerning MT and drug resistance but could also help explain how MT protects cells against other antineoplastic agents, such as doxorubicin, that are not formally electrophilic. In addition, protection from oxidative damage may not reside only in MT but may be a function of the associated metal, such as Zn, or other as yet undetermined proteins associated with MT.

METALLOTHIONEIN AS AN ANTIOXIDANT

Support for the hypothesis that MTs participate in intracellular defense against reactive oxygen and nitrogen species is derived from observations that MT genes are transcriptionally activated in cells and tissues during oxidative stress and that overexpression of MT reduces the sensitivity of cells and tissues to free radical–induced injury (68).

Induction of Metallothionein Expression in Prooxidant Conditions

A variety of physical and chemical stresses known to be associated with oxidative injury increase MT gene expression. X-irradiation (69), hyperoxia (70), and restraint (71) result in increased levels of oxygen free radicals and mRNA for MT in a tissue-specific fashion. Toxins, such as paraquat (4) and CCl_4 (72), and alkylating agents (3) and anticancer drugs, including doxorubicin, cisplatin, and bleomycin (3, 73, 74), have been reported to have similar effects in vivo. These and other stimuli result in an inflammatory response characterized by the production of cytokines, such as interleukin-1, interleu-

kin-6, tumor necrosis factor-α (TNF-α), and γ-interferon, that likely contribute to the induction of MTs (74–78). In addition, MT gene expression appears to be redox sensitive, for example: (*a*) UV irradiation induces MT mRNA in isolated Chinese hamster lung fibroblasts (2); (*b*) in *Saccharomyces cerevisiae*, the mRNA levels of the yeast MT counterpart, CUP1, are elevated in response to oxygen, heme, or growth conditions associated with production of oxygen free radicals (79); and (*c*) *tert*-butylhydroperoxide (tBH), which generates alkoxyl and peroxyl radicals, increases MT gene expression in NIH3T3 cells, perhaps via an AP-1 site in the 5′-flanking region of the MT gene (80).

Metallotheonein as a Free Radical Scavenger

Based on in vitro experiments, MTs can scavenge free hydroxyl and superoxide anions produced by xanthine-xanthine oxidase reaction (81) as well as inhibit degradation of isolated DNA by hydroxyl radicals (82). MT overexpression with heavy metals or cytokines produces resistance to oxidant injury caused by hyperoxia (83), ionizing radiation (56), CCl_4 (75), and hydrogen (84) and organic (85) peroxides. Nonetheless, Cd, Zn, and/or cytokines are promiscuous transcriptional activators and may affect aspects of cellular metabolism other than MT expression. For instance, it appears that glutathione and not MT is responsible for the heavy metal–associated resistance of V79 cells to H_2O_2 cytotoxicity (86).

Several genetic studies less encumbered by nonspecific induction artifacts reveal an antioxidant functionality for MT. *Saccharomyces cerevisiae* strains lacking a functional Cu-Zn-superoxide dismutase gene failed to grow on agar containing the respiratory carbon chain source lactate because of their enhanced sensitivity to oxidative injury (79). Expression of either yeast or simian MT after DNA transfer in the presence of Cu protected these mutants against oxygen free radical injury. Transfection of cells with a plasmid containing mouse MTI gene resulted in a fourfold increase in MT that was exclusively cytoplasmic. These cells were six times more resistant than isogenic controls to the cytotoxic effects of tBH (87). Overexpression of cytoplasmic MT did not protect against tBH-induced DNA damage, supporting the concept that membrane, not nuclear damage, is important in tBH cytotoxicity and suggesting that subcellular location might be important for its function. This latter concept was supported by observations that a clone of V79 cells with a high nuclear level of MT was resistant to H_2O_2 (88). Furthermore, embryonic cells cultured from MT knockout mice were more sensitive to Cd, tBH, and paraquat than wild-type cells (88a). Antisense oligonucleotides were used to lower nuclear MT and restore the sensitivity of V79 cells to the DNA damaging effects of H_2O_2 (88). Recently we have found that NIH3T3 cells that overexpress MT in the cytoplasm were fourfold more resistant to the cytotoxicity of the NO donor, S-nitroso-N-acetylpenicillamine (89), thus extending genetic

support for a protective role of MT against reactive nitrogen as well as oxygen injury. In this latter study, the presence of cytoplasmic MT was sufficient to limit the nuclear DNA strand breaks caused by highly diffusible NO species.

Mechanism of Action of Metallotheonein as an Antioxidant

The mechanism by which MT acts as an antioxidant remains unclear. In part, several contradictory studies underscore the complexity of the interaction of MT with reactive oxygen intermediates. For example, CHO cells that overexpress human MTIIA after gene transfer were not resistant to x-irradiation, bleomycin, or H_2O_2, although they were resistant to alkylating agents such as mitomycin C (22). The metal speciation of MT may, however, be critical because Cd-Zn MT actually induces DNA strand breaks in vitro (90) and because Cu MT was singularly protective against oxidant stress in mutant yeast (79). As noted above, both the cellular target for cytotoxicity and the subcellular location of MT may be critical, especially if MT is acting against diffusion-limited species, such as hydroxyl radical. The levels of other thiols, such as glutathione, may also be important for protection against oxidant injury by MT (84–86, 91). MTs can interact with glutathione (92) and MT can donate Cu to Cu-Zn superoxide dismutase (93).

MT may act as an expendable target for various reactive species. Thornalley & Vasak (81) originally suggested that cysteine residues were the primary target for interaction with hydroxyl radicals. Further studies indicated that release of Zn from MT might suppress the radical-mediated damage (94). Electron spin resonance data have shown that nitrogen oxide reactive species could be scavenged by iron-complexed MTs and/or thiol ligand exchange of iron-dinitrosyl moieties (89, 95). MT may sequester iron in such a fashion that it is no longer capable of contributing to Fenton chemistry (89, 96). The fate of MTs that have scavenged free radicals remains unknown, and thus it is unclear if such MTs are recycled, degraded, or affected in such a manner as to alter their metal-binding capacity.

CONCLUSIONS

MT was discovered as a result of its ability to bind to Cd. Although considerable information has been obtained on the structure of MTs and regulation of their expression, their physiological functions remain vague. In this review, we have summarized recent observations that MTs contribute to the resistance of mammalian cells to electrophilic (Table 1) and other (Table 2) antineoplastic agents. Although direct complexing of these drugs with MTs may be part of this phenomenon, the interaction of MT with existing or drug-induced secondary intracellular mediators may also be important (Figure 1). We hypothesize that the ability of MTs to act as expendable targets for oxygen, nitrogen, and

carbon-centered free radicals reduces the cytotoxic and nuclear-damaging effects of many of these agents. MTs are unusually diverse with respect to their isoform expression, metal speciation, and intracellular localization. This diversity underscores the attractiveness of MTs as pharmacological targets for enhancing the therapeutic index of anticancer drugs and may also contribute to current controversies regarding MTs and drug resistance. The recent development of genetically modified mice that lack functional MTI and -II (97, 98) provides an interesting new tool to examine the physiological role of MT. These mice appear to have a normal phenotype but are sensitive to heavy metals, such as Cd (97, 98). These knockout mice and their cells (88a) should help clarify the role of MT in protecting cells and organisms from the toxic actions of anticancer agents, oxidants, and mutagens.

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