Metallothionein Is a Potential Negative Regulator of Apoptosis

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Received December 3, 2002; accepted February 24, 2003

Apoptotic resistance can either be desirable or undesirable, depending on the conditions. In cancer chemotherapy, it is critical that tumor cells are selectively and effectively killed while leaving normal cells undamaged. Since acquisition of apoptotic resistance appears to be a common occurrence during malignant transformation, elucidating the mechanisms underlying apoptotic resistance is an area of intense study. Previous studies have revealed that metallothionein (MT) can protect cells from apoptosis induced by oxidative stress and metals. In the present study, we tested the hypothesis that the presence of MT may somehow modulate apoptosis. Our results revealed a strong linear negative correlation between basal MT levels and etoposide-induced apoptosis in the human tumor cell lines PLC/PRF/5, H460, and HepG2 (r = −0.991). In HepG2 cells, 24 h pretreatment with cadmium resulted in concentration-dependent increases in MT levels and marked decreases in etoposide-induced apoptosis. Zinc pretreatment also resulted in increased MT synthesis and decreased etoposide-induced apoptosis. More importantly, induced MT levels were negatively correlated with sensitivity to etoposide-induced apoptosis (r = −0.965). These suggest that MT may play a role in regulating apoptosis and that modulating MT expression may provide a strategy for altering cellular resistance to chemotherapeutic compounds.

Key Words: metallothionein; apoptosis; etoposide; cadmium; zinc.

Apoptosis is a cellular process by which damaged cells actively facilitate their own demise without damaging their neighbors, thus selectively removing themselves from the cellular population (Wyllie, 1992). Because of its selective nature and the fact that cells can be killed while leaving neighboring cells intact, induction of apoptosis is the preferred mode of action of cancer chemotherapeutics (Lowe and Lin, 2000). Consequently, factors that alter apoptotic responsiveness can either enhance the efficacy of such therapies or render the tumor cells resistant to treatment. The latter scenario is of utmost concern since acquisition of drug resistance is a major complication in cancer treatment. Similarly, acquisition of apoptotic resistance may be an important step in malignant transformation (Achanzar et al., 2000). Thus, identifying the mechanisms that can confer apoptotic resistance is of great importance because such knowledge may allow for the development of strategies to prevent or overcome drug resistance and define mechanisms of carcinogenesis. Consequently, it may also be possible to sensitize cells to chemotherapy by enhancing their apoptotic sensitivity.

Numerous factors can determine cellular sensitivity to drug-induced apoptosis. For instance, sensitivity can be dependent on cellular levels of proteins critical for regulating apoptosis. Tumor suppressor proteins, such as p53 and Bax, are pro-apoptotic and functional loss of these proteins can confer resistance to apoptotic stimuli (Chao and Korsmeyer, 1998; Hickman et al., 2002). Other proteins, including Bcl-2, are anti-apoptotic and overexpression confers apoptotic resistance while decreased expression or loss promotes cell death (Chao and Korsmeyer, 1998). Another factor that influences sensitivity to drug-induced apoptosis is the ability of cells to either sequester or efflux chemotherapeutic agents. This cellular strategy underlies the multidrug resistant phenotype often observed in cancer, where overexpression of drug efflux pumps (such as MDRs and MRPs) in tumor cells confers resistance to a variety of compounds.

A potentially important regulator of apoptosis is metallothionein (MT). MT is actually a family of small (6–7 kDa) metal-binding proteins that are highly conserved evolutionarily (Klaassen et al., 1999). The first recognized function of MT was detoxification of heavy metals such as cadmium and mercury (Klaassen et al., 1999). Consequently, factors that alter apoptotic responsiveness can either enhance the efficacy of such therapies or render the tumor cells resistant to treatment. The latter scenario is of utmost concern since acquisition of drug resistance is a major complication in cancer treatment. Similarly, acquisition of apoptotic resistance may be an important step in malignant transformation (Achanzar et al., 2000). Thus, identifying the mechanisms that can confer apoptotic resistance is of great importance because such knowledge may allow for the development of strategies to prevent or overcome drug resistance and define mechanisms of carcinogenesis. Conversely, it may also be possible to sensitize cells to chemotherapy by enhancing their apoptotic sensitivity.

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2002), and cisplatin in mouse embryonic cells (Kondo et al., 1997). The basis of these results is likely due to direct interactions of the apoptosis-inducing agents (e.g., metals) with the MT protein that, in effect, lowers the free concentration of the apoptotic agent.

Cadmium is a toxic heavy metal with pro-apoptotic potential in various cells in vivo and in vitro (Achanzar et al., 2000; Habeebu et al., 1998). However, several studies have demonstrated that cadmium can also be anti-apoptotic in some circumstances (Shimada et al., 1998; von Zglinicki et al., 1992; Yuan et al., 2000). Similarly, zinc exposure can block apoptosis in several cases (Leccia et al., 1999; Meerarani et al., 2000; Shimoda et al., 2001). Cadmium and zinc are both effective inducers of MT synthesis and bind avidly to the protein (Andrews, 2000), thus one untested hypothesis is that the anti-apoptotic effects of these metals may be related to MT. The present study aimed to test this hypothesis by examining the effect of MT on etoposide-induced apoptosis in a number of human cell lines. Etoposide was selected because it is an apoptotic agent. The specific aims of this study were to assess the effective inducer of apoptosis and is not known to associate with MT. The specific aims of this study were to assess the relationship between basal cellular MT levels and etoposide-induced apoptosis in various cell lines and to investigate the effect of induced levels of MT on etoposide-induced apoptosis.

MATERIALS AND METHODS

**Chemicals.** Cadmium chloride (CdCl₂), zinc chloride (ZnCl₂), MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide), and etoposide were obtained from Sigma (St. Louis, MO).

**Cell lines and culture conditions.** The human hepatoma cell lines PLC/PRF/5 and HepG2 and the human lung large cell carcinoma cell line NCI-H460 were purchased from the American Type Culture Collection (Manassas, VA). The cells were maintained at 37°C in a humidified 5% CO₂ atmosphere and cultured as monolayers in Dulbecco’s modified Eagle’s medium (DMEM; PLC/PRF/5 and HepG2) or RPMI-1640 (NCI-H460; Life Technologies, Gaithersburg, MD) supplemented with 10% fetal bovine serum, 31 U/ml penicillin G, and 50 µg/ml streptomycin (Life Technologies).

**MT quantitation.** Cellular MT concentrations were estimated by the cadmium-hemoglobin radioassay method as described by Eaton and Toal (1982). MT was measured in untreated cells or in cells treated with cadmium, zinc, or etoposide at the designated concentrations.

**Quantification of apoptotic DNA fragmentation.** Apoptotic DNA fragmentation was assessed by quantitative determination of cytoplasmic histone DNA-fragments using the Cell Death Detection ELISA kit (Roche, Indianapolis, IN). In all cases, cells were seeded in 96 well plates at 1 × 10⁴ cells/well in 100 µl medium and treatments initiated 24 h after plating. To examine the effects of basal MT levels on apoptosis, the medium was replaced with 100 µl fresh medium with or without 100 µg/ml etoposide and apoptosis was evaluated in both floating and adherent cells 20 h later. To examine the effect of induced MT, the cells were pretreated for 24 h with either cadmium (0–2 µM) or zinc (100 µM) at concentrations to not induce significant cytotoxicity as assessed by the MTT assay (Mosmann, 1983). The medium was then removed and replaced with 100 µl of metal-free medium with or without 100 µg/ml etoposide and apoptosis was evaluated in both floating and adherent cells 20 h later.

**Quantification of caspase-3 activity.** HepG2 cells were pretreated with 0–2 µM cadmium 24 h, after which the medium was replaced with metal-free medium containing 75 µg/ml etoposide. Caspase-3 activity was evaluated 16 h later using an Apoalert Caspase-3 Fluorescent Assay Kit (Clontech, Palo Alto, CA). Briefly, 1 × 10⁴ cells were lysed and the lysates were incubated with a conjugated caspase-3 substrate that normally fluoresces at 405 nm, but proteolytic cleavage by caspase-3, fluoresces at 505 nm. Data are expressed as percent of caspase-3 activity in untreated control cells (p < 0.05).

**Statistics.** Data are given as the mean ± SEM unless otherwise noted. Results were analyzed using Student’s t-test or Dunnett’s or Tukey’s multiple comparison tests after ANOVA as appropriate. Correlation between apoptotic rates and basal- or induced-MT concentrations was tested by calculating Pearson’s correlation coefficient, r. In all cases, the level of significance was set at p < 0.05.

RESULTS

Genomic DNA fragmentation was used as an indicator of apoptosis and was determined by using an ELISA-based assay that measures cytoplasmic nucleosomes. As seen in Figure 1, exposure to 100 µg/ml etoposide for 18 h resulted in significant DNA fragmentation in the three cell lines investigated, with PLC/PRF/5 cells being most sensitive and HepG2 being the least sensitive. Similarly, when cell viability was assessed by the ability to metabolize MTT, PLC/PRF/5 cells were found to be most sensitive to etoposide-induced cytolethality while
HepG2 cells were most resistant (data not shown). To determine if cellular MT levels were related to etoposide-induced apoptosis or cytolethality, basal MT levels were determined for each cell line (Fig. 2). The basal MT content was lowest in PLC/PRF/5 cells, intermediate in H460 cells, and highest in HepG2 cells. When the correlation between the basal level of cellular MT and etoposide-induced DNA fragmentation was then examined. As shown in Figure 5, cadmium pretreatment resulted in significant inhibition of etoposide-induced DNA fragmentation rate in a cadmium concentration-dependent manner. In fact, at the highest cadmium concentration (2 μM), DNA fragmentation was reduced by 56.4% compared to the cells without cadmium pretreatment. Similar results were obtained when MT synthesis was induced by zinc pretreatment (data not shown). Pretreatment of HepG2 cells with 100 μM zinc for 24 h resulted in an approximately threefold increase in cellular MT levels. More importantly, these zinc-pretreated cells exhibited a 36% reduction in etoposide-induced apoptosis compared to cells pretreated with medium only.

The relationship between induced levels of MT and etoposide-induced DNA fragmentation rates was then analyzed. As was observed with basal MT levels, there was a significant negative correlation between cadmium-induced MT levels and etoposide-induced DNA fragmentation (r = −0.965, p < 0.05; Fig. 6). As a secondary measure of apoptosis, we investigated the effect of cadmium pretreatment on etoposide-induced activation of caspase-3 in HepG2 cells. In agreement with the DNA fragmentation data, caspase-3 activity was significantly decreased by 24 h pretreatment with 1.0 and 2.0 μM cadmium (Fig. 7). When the relationship between etoposide-induced caspase-3 activity and cadmium-induced MT synthesis was examined, a significant negative correlation was observed (r = −0.863, p < 0.05).

**DISCUSSION**

Due to the pivotal role of apoptosis in cancer, MT-induced apoptotic inhibition has important implications for both carcinogenesis and cancer therapy. In the present study, we clearly demonstrated that MT can protect cells from apoptosis induced by the anti-cancer drug etoposide. While it is well established that MT expression (or overexpression) can protect cells from apoptosis stemming from uncorrected DNA damage.

It is well known that cadmium can be a very effective inducer of MT synthesis, so we investigated the effect of cadmium induction of MT on etoposide-induced apoptotic cell death. When HepG2 cells were pretreated for 24 h with 0.25–2 μM cadmium, a linear dose-dependent increase in MT levels was observed (Fig. 4). These levels of cadmium did not result in significant cytotoxicity in HepG2 cells (not shown). The effect of cadmium pretreatment on etoposide-induced apoptosis was then examined. As shown in Figure 5, cadmium pretreatment resulted in significant inhibition of etoposide-induced DNA fragmentation rate in a cadmium concentration-dependent manner. In fact, at the highest cadmium concentration (2 μM), DNA fragmentation was reduced by 56.4% compared to the cells without cadmium pretreatment. Similar results were obtained when MT synthesis was induced by zinc pretreatment (data not shown). Pretreatment of HepG2 cells with 100 μM zinc for 24 h resulted in an approximately threefold increase in cellular MT levels. More importantly, these zinc-pretreated cells exhibited a 36% reduction in etoposide-induced apoptosis compared to cells pretreated with medium only.

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**FIG. 2.** Basal metallothionein (MT) levels in PLC/PRF/5, H460, and HepG2 cells. Data represented as mean (n = 3). Bars, SE. *Significant difference from PLC/PRF/5 (p < 0.05).

**FIG. 3.** Correlation between sensitivity to etoposide-induced apoptosis (Fig. 1) and basal MT levels (Fig. 2). Data represented as mean (n = 3). Bars, SE for both x- and y-axis values.
a variety of apoptotic stimuli, including chemotherapeutic agents (Kondo et al., 1997; Wang et al., 2001), our work is distinctive in that it shows that the anti-apoptotic effects of MT are highly dependent on cellular MT levels. For many agents, such heavy metals and oxidative radicals, the primary protective mechanism is sequestration by MT (Klaassen et al., 1999; Lazo et al., 1998). Etoposide is not known to interact with MT and, while there is evidence that MT can protect cells from the toxic effects of chemicals that are either not bound by MT or at least not bound tightly (Klaassen et al., 1999), the strong correlation between MT concentration and resistance to etoposide-induced apoptosis is surprising. The fact that this correlation is apparent with both basal and induced MT levels indicates that MT itself may be an important factor in mitigation of apoptosis. Since etoposide induces apoptosis at a very basic level, namely through uncorrected DNA damage, MT appears to play a generalized role in prevention of apoptosis.

Overexpression of MT has been reported for a wide variety of tumors (Jasani and Schmid, 1997) and cellular MT levels have been correlated with increasing tumor grade in cancers of
the prostate, kidney, breast, lung, and ovary (Jin et al., 2002; Moussa et al., 1997; Tan et al., 1999; Theocharis et al., 2002; Tuzel et al., 2001). More significantly, increased MT expression appears to be a marker of poor prognosis in a number of cancers, including colorectal cancer, breast cancer, lung cancer, and renal cancer (Janssen et al., 2002; Joseph et al., 2001; Sens et al., 2001; Tuzel et al., 2001). Defective or diminished apoptosis is well known to be important in tumorigenesis, and it is clear that many tumor cells possess either inherent resistance to chemotherapeutic agents or acquire resistance after initial exposures (Johnstone et al., 2002). In this regard, several in vitro studies have illustrated the importance of MT expression for tumor cell growth and survival. Takeda et al. (1997) demonstrated that antisense-mediated downregulation of MT protein production resulted in dramatic growth inhibition in several human cancer cell lines. In MCF-7 human breast cancer cells, the downregulation of MT production using antisense oligonucleotides not only inhibited cell growth but also precipitated spontaneous apoptotic cell death (Abdel-Mageed and Agrawal, 1997). Similarly, when MT expression was abolished in human PC-3 prostate and SKOV-3 ovarian cancer cell lines using a specific ribozyme, there is a marked increase in spontaneous apoptosis (Tekur and Ho, 2002). Consistent with the present results showing MT inhibits apoptosis, these studies indicate that MT may play a role in determining sensitivity of tumor cells to apoptosis.

The present study clearly shows that MT is effective at protecting cells from etoposide-induced apoptosis and that this protection is directly dependent on the level of cellular MT. The current results also show that the inhibitory role of MT is not dependent on the cell type, at least with the cell lines used, and occurs regardless of whether one considers basal or induced MT. MT is certainly not the only factor that determines apoptotic sensitivity and there are a large number of cellular factors, such as glutathione levels and p53 status, that could have contributed to the observed differences in apoptosis. For instance, the three cell lines we used may exhibit differences in the expression of pro- or anti-apoptotic genes such as bcl-2, bax, and p53, all of which are known to have profound effects on apoptotic sensitivity. Even if such differences exist, it does not diminish our observation of a strong negative correlation between levels of MT and apoptosis. With regard to the experiments where MT was induced by cadmium, it is possible that the cadmium treatment could have enhanced expression of other genes important in apoptosis. Cadmium-induced activation of both oncogenes and tumor suppressor genes has been reported previously (Achanzar et al., 2000; Jin and Ringertz, 1990; Matsuoka and Call, 1995). However, when apoptosis was examined, it was found that cadmium-induced oncogene and tumor-suppressor gene expression was correlated with induction of apoptosis rather than inhibition (Achanzar et al., 2000; Matsuoka and Call, 1995).

Given that MT is unlikely to bind etoposide directly, the question becomes what is the mechanism underlying this protective effect. It has been previously shown that overexpression of MT can protect cardiomyocytes from the apoptotic effects of doxorubicin (Wang et al., 2001). Further investigation revealed that MT overexpression inhibited doxorubicin-induced generation of reactive oxygen species (ROS; Wang et al., 2001). Etoposide is a potent inhibitor of DNA topoisomerase II that prevents DNA ligation and ultimately leads to formation of DNA strand breaks (D’Arpa and Liu, 1989). When formation of ROS during etoposide-induced apoptosis was examined, only slight increases were observed and apoptosis was not inhibited by antioxidants (Garcia-Bermejo et al., 1998). More recently, work in HL-60 cells demonstrated the formation of etoposide-derived phenoxyl radicals that can oxidize glutathione and, when cellular glutathione is depleted, protein sulfhydryl groups (Kagan et al., 2001). Thus, one possible hypothesis to explain MT inhibition of etoposide-induced apoptosis is that MT, being sulfhydryl-rich, acts as a scavenger for these phenoxyl radicals and prevents them from interacting with more critical cellular proteins. Consequently, cells with more MT would presumably be less sensitive to the detrimental effects of these radicals. However, there is no direct evidence that MT can interact with etoposide-induced radicals. Indeed, etoposide can also act as an anti-oxidant and the oxidative radicals it
produces are not reactive enough to trigger lipid peroxidation (Kagan et al., 2001). So, direct sequestration of etoposide or an etoposide metabolite seems unlikely as a complete explanation for the inhibitory effects of MT on apoptosis observed in the current study.

An alternative hypothesis is related to the ability of zinc to inhibit apoptosis. Zinc is a potent inhibitor of caspase-3 (Perry et al., 1997) and the calcium-dependent endonuclease (Lohmann and Beyersmann, 1993), both of which are critical for apoptotic cell death. Moreover, depletion of zinc by chelation has been shown to promote apoptosis in vitro (Chimienti et al., 2001). Under this hypothesis, either the ambient release of zinc from MT and/or etoposide-derived radical-mediated oxidation of sulfhydryl groups causes release of zinc from MT, and the free zinc then can inhibit apoptosis. This hypothesis accounts for the observation that apoptotic inhibition is dependent on MT levels as more zinc would be liberated in cells containing more MT. Similarly, nitric oxide, a compound shown to exert anti-apoptotic effects in a number of cell types (Kim et al., 2001), has been shown to displace zinc from MT (Katakai et al., 2001; St. Croix et al., 2002). Thus, zinc bound to MT may play an important role in the control of apoptosis, although further work will be required to establish this as a critical factor.

One possible complicating factor in the present study is that both cadmium (Shimada et al., 1998; Yuan et al., 2000) and zinc (Fukamachi et al., 1998; Leccia et al., 1999; Meerarani et al., 2000) have been shown to protect cells from apoptosis, making it possible that the apoptotic resistance observed after cadmium or zinc pretreatment may be due to the direct effects of metals rather than induced MT. For instance, cadmium can directly inhibit caspase-3, although the 50% inhibitory concentration (IC50) in cell free systems is approximately 30 μM (Yuan et al., 2000), which is 15 times the highest cadmium concentration (2 μM) used in the present study. Furthermore, it would appear unlikely that the metals were directly inhibiting apoptosis since, following metal pretreatment, the etosipide exposures were performed in metal-free medium and, given the marked increases in MT, there was probably relatively little free metal present at the time apoptosis was induced by etosipide. Additionally, the results observed with induced MT levels were very similar to those from the experiment comparing basal MT levels and apoptosis in that it was MT protein that correlated with diminished apoptosis induced by etosipide. Thus, direct cadmium or zinc effects in blocking some critical step in dedication to apoptosis after etosipide seems unlikely.

The finding that apoptotic inhibition is dependent, at least in part, on cellular MT levels has important implications for chemotherapeutic strategies. A major problem in cancer chemotherapy is that the drugs often have adverse effects that are related to secondary toxicities. Since MT expression is inducible by a number of stimuli, such as metals and glucocorticoids (Klaassen et al., 1999), it may be possible in the future to develop compounds that will stimulate MT synthesis in specific tissues, thereby reducing or preventing unwanted toxicities. Conversely, MT expression could be repressed specifically in tumors, making them more sensitive to induction of apoptosis by chemotherapeutic agents. Such downregulation could be achieved using pharmacologically targeted adaptations of the antisense or ribozyme methods or through the use of chemical agents such as clofibrate acid, which was recently shown to induce downregulation of MT in human hepatoma cells (Bianchi et al., 2002).

In summary, the present results demonstrate that cellular MT can be an effective inhibitor of etoposide-induced apoptosis. This inhibition occurred regardless of the precise cell line employed and regardless of whether basal or induced MT was considered. The role of MT in control of apoptosis warrants further investigation.

REFERENCES


