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ZINC EFFECTS ON GLUTATHIONE METABOLISM RELATIONSHIP TO ZINC-INDUCED PROTECTION FROM ALKYLATING AGENTS*

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Abstract—Several aspects of the effects of zinc on the metabolism of glutathione were examined in the Chinese hamster cell (line CHO) and in three derived sublines which differ in their resistance to the thiol reactive heavy metal cadmium. In the parental CHO cell, which does not induce the synthesis of metallothionein in response to zinc, glutathione levels remained approximately constant during the first 6 hr of zinc exposure. In the resistant cell lines, which induce the synthesis of metallothionein in response to zinc, the glutathione levels dropped transiently during zinc exposure. In all cell lines except the most cadmium resistant line, the glutathione levels after 12 hr were increased up to 3-fold relative to pretreatment levels. Similarly, the glutathione *S*-transferase activity measured by the conjugation of 1-chloro-2,4-dinitrobenzene to glutathione was increased after 9–12 hr of zinc treatment in all except the most highly cadmium resistant cell line. Glutathione reductase was not affected consistently by zinc treatment; however, the level of activity of this enzyme in the most highly cadmium resistant line was two to three times greater than that observed in the other cell lines. These effects are considered in relation to the zinc-induced protection of these cells from the toxic effect of the alkylating agent melphalan.

Zinc is an important trace metal nutrient which is required for the activity of over thirty well-characterized enzymes [1]. It is also an effective inducer of the small cysteine-rich proteins known as metallothioneins (MTs)‖, which bind metal ions and are implicated as an intracellular reserve for Zn^{2+} [2, 3] and Cu^{2+} [4, 5]. The importance of studying other cellular responses to high levels of zinc was indicated by recent reports by Tobey *et al.* [6]. Their studies demonstrated that zinc pretreatment protects Chinese hamster cells in culture from the toxic effects of melphalan (phenylalanine mustard), an alkylating agent which is employed in cancer chemotherapy [7]. Because alkylating agents have a high reactivity toward thiol groups, it was postulated initially that the zinc-induced protection might result from reaction of melphalan with the thiol-rich MTs. However, the CHO cell line has been shown to be deficient in MT production [8], but was protected by zinc pretreatment at least as much as cell lines which do induce MT when treated with zinc. These findings suggested that an investigation should be undertaken to define other effects of zinc on cellular metabolism and, in particular, metabolic processes involving those thiol-containing components which might modulate alkylating agent toxicity.

One important mechanism for cellular detoxification of electrophilic agents involves their enzymatic conjugation with glutathione (GSH) [9], an important cellular thiol compound. In this context, Cagen and Klaassen [10] showed that GSH levels in rat liver were depleted by several alkylating agents (diethylmaleate, iodomethane, and iodoacetate). On the other hand, Wong and Klaassen [11] found that zinc increased the GSH levels in rat liver. Finally, Chung and Maines [12] showed that selenium, another essential trace element which protects the cells from melphalan [13], increased the rate-limiting step of GSH synthesis (γ-glutamylcysteine synthetase) as well as the activity of glutathione disulfide (GSSG) reductase.

These previous studies suggest that metal-induced alterations in GSH metabolism may play a role in the zinc-induced protection of the cells from melphalan. This paper presents an investigation of the effects of zinc on cellular GSH levels and on the activity of two enzymes, GSH *S*-transferase and GSSG reductase, in four cell lines which differ greatly in cadmium resistance, in ability to induce MT synthesis, and zinc-induced protection from melphalan. The relationships among metal metabolism, glutathione metabolism, and melphalan toxicity are discussed.

MATERIALS AND METHODS

Glutathione reductase, GSH, GSSG, 1-chloro-2,4-dinitrobenzene, 5,5′-dithiobis-(2-nitrobenzoic acid), and NADPH were products of the Sigma Chemical Co., St. Louis, MO. Melphalan was a gift from Dr. David Abraham, Investigational Drug Branch, NCI, Bethesda, MD. All other reagents were reagent grade or better.

Stock solutions of 1 M $ZnCl_2$ were prepared in

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‖ Abbreviations: MT, metallothionein; GSH, glutathione, reduced form; and GSSG, glutathione, oxidized form.

Table 1. Protection from melphalan toxicity by zinc*

Cell line	Time in 16 μM melphalan: % Survival (0 hr)	% Survival (2 hr)	Relative protection
CHO	96.5	0.91	
CHO + Zn^{2+}	94.5	13.4	14.7×
Cd^r20F4	92	0.43	
Cd^r20F4 + Zn^{2+}	93.5	3.0	7.0×
C^r30F9	91	0.23	
Cd^r30F9 + Zn^{2+}	89.5	1.20	5.2×
Cd^r200T1	101.5	0.026	
Cd^r200T1 + Zn^{2+}	97.5	0.043	1.7×

* Cells were treated with 110 μM $ZnCl_2$ or sham-treated with water for 9 hr. Thereafter, aliquots were plated (t = 0 hr), and the remaining cells were treated with 16 μM melphalan for 2 hr and then plated in drug-free medium. After 1 week, the colonies (50 or more cells) were stained with crystal violet and counted.

0.1 N HCl, sterilized by filtration, calibrated by atomic absorption spectrometry, and stored at −20°.

Several Cd^{2+}-resistant sublines of the Cd^{2+}-sensitive CHO cell were derived as previously described [14]. The Cd^{2+}-resistant sublines used in this study were cloned at Cd^{2+} exposure levels of 20, 30, and 200 μM $CdCl_2$ in monolayer culture [14, 15]; these Cd^{2+}-resistant sublines were designated Cd^r20F4, Cd^r30F9, and Cd^r200T1 respectively. All were shown to maintain their resistance to Cd^{2+} in the absence of selective pressure. The cadmium resistance appears to be largely a function of the ability of the cells to induce the synthesis of MTs in response to a challenge with metal ions. These Cd^{2+}-resistant cell lines also increase synthesis of MT in response to treatment with zinc [15, 16].

For these experiments, the cells were grown in suspension culture in F-10 medium supplemented with 15% neonatal calf serum as previously described [8]. Survival studies (colony-forming ability) were performed as described elsewhere [6].

The metabolic studies were performed on exponentially growing cells treated with 110 μM $ZnCl_2$ for various periods. Cytoplasm fractions were prepared as previously described [8], except that dithiothreitol was omitted. Samples that were to be analyzed for enzyme activities contained 20 mM β-mercaptoethanol.

Glutathione content (GSH + GSSG) was measured by a modification of the method of Teitze [17]. The proteins were precipitated with 0.1 M perchloric acid and removed by centrifugation. The supernatant fraction was neutralized with KOH and frozen slowly to accelerate precipitation of the potassium perchlorate. After centrifugation to remove the precipitate, the supernatant fraction was acidified to 0.01 N HCl to protect the GSH from disulfide exchange with any other sulfhydryl compounds remaining in the preparation. A standard curve was constructed using GSSG.

Glutathione reductase activity was assayed according to Staal *et al.* [18]. GSH *S*-transferase activity was measured by the method of Habig *et al.* [19] using 1 mM 1-chloro-2,4-dinitrobenzene as the substrate. Both reaction mixtures contained 0.2 mM EDTA to protect the enzymes from inhibition by metal ions (unpublished results; [20, 21]). All assays were performed in a volume of 1 ml in a Perkin–Elmer model 552 spectrophotometer, with the sample cuvette maintained at 25° with a heating block.

Protein was measured by the method of Bradford [22] using commercial reagent (BioRad). Enzyme activities were reported as percent change in specific activity (μmoles product/min/mg protein), and GSH + GSSG content was reported as percent change in μg GSH + GSSG/mg protein.

RESULTS

The results of experiments examining the effect of zinc pretreatment on the cytotoxicity of melphalan in the CHO cell and in the MT-proficient Cd^r lines are shown in Table 1. These results, showing a zinc-induced protection against melphalan toxicity in the CHO, Cd^r20F4, and Cd^r30F9 cells, confirm previous measurements [6]. The protective phenomenon was also observed here in a recently derived MT-overproducing cell line, the Cd^r200T1 cells [15]. This cell line grows normally in 100 μM $CdCl_2$ (in suspension culture), displays constitutive MT synthesis in the absence of exogenous Cd^{2+} or Zn^{2+} at a rate comparable to the fully Cd^{2+} or Zn^{2+}-induced Cd^r30F9 cells, and can be induced by Cd^{2+} or Zn^{2+} to synthesize MT at a rate approximately 10-fold greater than the constitutive level (data not shown). It is unexpectedly sensitive to melphalan and is not protected to the extent of the other cell lines by pretreatment with zinc.

If GSH is important in the zinc-mediated protection from melphalan in these cells, there should be a correlation between the levels of GSH and the degree of protection (increased survival) in the zinc-treated cultures. Results of experiments to reveal such a correlation are shown in Fig. 1. It is apparent that, during the first 6 hr following addition of $ZnCl_2$, the GSH concentration in the various cells either remained essentially unchanged (CHO cells)

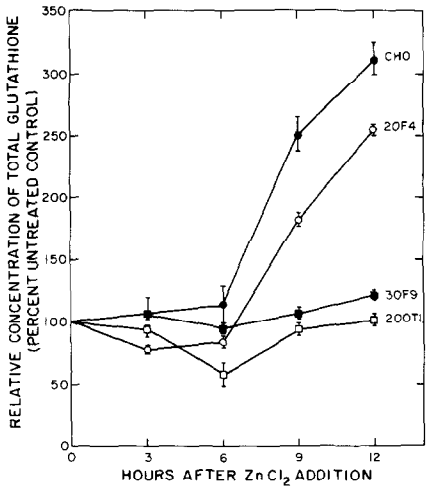

Fig. 1. Induction of total glutathione by 110 μM $ZnCl_2$. Cells were treated with 110 μM $ZnCl_2$ beginning at 0 hr. At 3-hr intervals, aliquots were removed and assayed for total glutathione as described in the text.

or decreased somewhat. Following this, the concentration in all cell lines (except Cd^{r}200T1 in the experiment shown) increased to levels greater than the controls, although the magnitude of the increase varied among the cell lines. In other experiments, the 12-hr GSH level in Cd^{r}200T1 cells was as high as 127% of the controls, i.e. comparable to the Cd^{r}30F9 cells but well below the levels in the CHO and Cd^{r}20F4 cells.

Other possible mechanisms for increasing the rate of detoxification of the drug could be increased GSH *S*-transferase activity or increased concentration of reduced GSH. The GSH *S*-transferase and GSSG reductase activities were measured in preparations of cells from cultures that had been exposed to 110 μM $ZnCl_2$ for 0, 3, 6, 9, 12 or 24 hr. The effects of $ZnCl_2$ treatment on GSH-transferase activity in the different cell lines are shown in Table 2. There was some variability in the time course and extent of induction in the various cell lines. In particular, in later experiments, a higher concentration of $ZnCl_2$ was required to induce the same levels of enzyme activity. In general, however, the enzyme activity in the CHO, Cd^{r}20F4, and Cd^{r}30F9 cell lines appeared to increase, while that in the Cd^{r}200T1 cells did not, or did so to a smaller extent.

Glutathione reductase, on the other hand, was affected little during the course of zinc treatment, and the variations were not reproducible. Although zinc did not appear to alter the activity of this enzyme within any line, there were significant differences in the constitutive levels of glutathione reductase activity among cell lines. The specific activity of glutathione reductase in the Cd^{r}200T1 cells (72 ± 18 μmoles/min/mg) was approximately twice that of the other cell lines (38 ± 11, 32 ± 16 and 37.4 ± 1.4 μmoles/min/mg for CHO, Cd^{r}20F4 and Cd^{r}30F9 respectively).

DISCUSSION

In an earlier report, Tobey *et al.* [6] concluded that zinc-induced synthesis of MT alone could not be responsible for the protection of the cells from melphalan. The conclusion was based on the fact that CHO cells are clearly protected from melphalan cytotoxicity by zinc while no measurable increase in MT synthesis occurs. The observations of these authors that CHO cells, as well as Cd^{r}20F4 and Cd^{r}30F9 cells, are protected by $ZnCl_2$ pretreatment are confirmed in this study, and the conclusion is strengthened by the result in the Cd^{r}200T1 cells. Although these cells constitutively synthesized MT at a rate comparable to maximally induced Cd^{r}20F4 or Cd^{r}30F9 cells, they were about 30-fold more sen-

Table 2. Induction of GSH *S*-transferase activity by $ZnCl_2$*

Cell line	Experiment No.	[$ZnCl_2$] (μM)	Time of maximum activity	% of t = 0 activity
CHO	1	100	12	132
	2	100	12	101
	3	110	12	117
	4	110	12	125
20F4	1	100	12	131
	7	110	12	137
30F9	1	100	12	124
	2	100	12	106
	4	110	9	117
	6	110	12	132
200T1	2	100	12	121
	5	110	9	108
	8	110	9	97.5†

* Cells in suspension culture were treated with the indicated concentrations of $ZnCl_2$ for 0, 3, 6, 9, 12 or 24 hr before preparation for assay. Assays were performed within 1 hr as described in the text.

† Activity dropped, then recovered, then dropped again. The reported value is the maximum of the recovery.

sitive to melphalan than were CHO cells. Furthermore, although zinc caused an approximately 10-fold further increase in MT synthesis in the Cd^{r}200T1 cells, there was little zinc-induced protection from melphalan.

Having eliminated zinc-induced MT as a candidate for this protective mechanism, we examined the GSH conjugation pathway. Since many drugs and other xenobiotic compounds are known to be metabolized through GSH *S*-transferase-catalyzed conjugation with GSH, this potential detoxification mechanism was examined from the viewpoint of both the enzyme and substrate levels during zinc treatment in each of the cell lines (Fig. 1 and Table 2).

The results show that zinc increased the levels of GSH in at least three of the cell lines (the exception being the Cd^{r}200T1 cells), and that the degree of protection was roughly proportional to the induction of GSH in the various cells. This increase in GSH level was preceded by a decrease in all of the cadmium-resistant, MT-synthesizing cell lines, but not in the CHO parent line. Since it is known that one important factor regulating GSH synthesis is the availability of cysteine [23], we propose that the initial decrease in GSH could have been a consequence of depletion of the cysteine pools by the rapid synthesis of the cysteine-rich MTs in the Cd^r cell lines. The absence of a decrease in GSH level in CHO cells, which do not synthesize MT during exposure to zinc, supports this idea (Fig. 1).

It is tempting to speculate that the increase in GSH levels in the Cd^{r}20F4 and Cd^{r}30F9 cell lines from 6 to 12 hr after addition of $ZnCl_2$ might be explained by an overcompensation by the cells to these initially depressed cysteine levels. However, the observation that the CHO cell shows the greatest increase in GSH during $ZnCl_2$ treatment in the absence of the initial decrease suggests that a simple overcompensation cannot explain this phenomenon. An alternative hypothesis is that GSH binds Zn^{2+} and that this effectively lowers the free GSH concentration, signalling a need for GSH synthesis.

An increase in the level of GSH could, in itself, cause an increase in non-enzymatic conjugation to electrophiles by mass action. However, since the cellular concentration of GSH is normally well above its K_m for GSH *S*-transferase [24] (K_m is the substrate concentration giving half-maximal activity), even large increases in the concentration could produce only relatively small increases in the rate of the enzymatic reaction. On the other hand, increases in the GSH levels prior to the addition of a GSH-depleting drug would minimize the decreases in reaction rate as the GSH was depleted. Furthermore, increased synthesis of GSH (presumably the cause of the increased concentration) would replace the conjugated GSH more rapidly. Finally, these arguments do not take into account any distribution of GSH into pools. It is possible that the GSH pool available for conjugation is only a part of the total GSH, in which case increases in this pool could have a large effect on the reaction rate.

The changes in the GSH *S*-transferase activity are quantitatively much smaller than those in the GSH levels. However, changes in enzyme activity affect the reaction rate directly. In this respect, it is important to note that the assay employed in this study uses 1-chloro-2,4-dinitrobenzene as the substrate, as it is a generally acceptable substrate for the known GSH *S*-transferases [19]. Thus, the measured activity is a composite activity of several enzymes with distinct substrate specificities. It has been demonstrated previously that several relatively non-specific drugs caused different effects on the various GSH *S*-transferase activities [25]. If melphalan is a substrate for one of the transferases and zinc is a specific activator for that isozyme, a large change in that particular transferase could result in a much smaller change in the measured activity. That is, a small change in the measured activity could reflect a major change in the ability of the cell to conjugate and detoxify the drug.

Traces of heavy metals may catalyze the autooxidation of GSH to GSSH [26]. Since the cellular defense against oxidation of GSH is the activity of GSSG reductase, it was of interest to examine this activity after zinc exposure. In the experiments reported here, zinc had no consistent effect on GSSG reductase activity. However, it was observed that the Cd^{r}200T1 cells had a constitutive level of GSSG reductase two to three times the levels found in CHO, Cd^{r}20F4, and Cd^{r}30F9 cell lines. These findings suggest that long-term culture in increasing cadmium concentrations may have caused this alteration in GSH metabolism. Although zinc exposure did not alter significantly GSSG reductase activity in those experiments, Chung and Maines [12] observed that the activity of this enzyme increased in rat liver following the administration of selenite to rats. The enzyme activity increased approximately 25% in 24 hr, following a transient increase in GSSG. This observation is of interest since selenite, like zinc, provides some protection against melphalan in CHO cells [13].

The results presented here may have useful consequences for alkylating agent chemotherapy. There is already evidence for the role of cellular thiols in protecting the cell from some toxic agents [27, 28] and radiation [29, 30]. In particular, Boyd *et al.* [31] showed that depletion of GSH sensitized lung tissue to the toxicity of another alkylating agent, 4-ipomeanol, and that when the activation of the drug was inhibited, the depletion of glutathione was decreased. The studies reported here complement these previous studies, showing that increases in GSH and GSH *S*-transferase are correlated with protection against melphalan. While proof of the mechanism of zinc-induced protection awaits direct measurement of increased conjugation of melphalan to GSH, these studies clearly support the hypothesis that zinc induces the GSH *S*-transferase pathway. It is possible that manipulation of the level of zinc, a nutritionally important trace element, could be used to induce the protection and lead to improved chemotherapeutic regimens.

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