Glutathione Depletion as a Determinant of Sensitivity of Human Leukemia Cells to Cyclophosphamide

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ABSTRACT

The role of glutathione (GSH) as a determinant of cellular sensitivity to the cytotoxic and DNA-damaging effects of cyclophosphamide (CP) was studied in a dual culture system of rat hepatocytes and K562 human chronic myeloid leukemia cells, which have elevated aldehyde dehydrogenase activity with a corresponding insensitivity to activated CP.

Exposure of K562 cells to 50 μM dl-buthionine-S,R-sulfoximine for 24 h resulted in a depletion of cellular GSH content to 10% of control values without toxicity. Subsequent 1-h exposure of GSH-depleted cells to activated cyclophosphamide, obtained by incubation of CP with suspension cultures of rat hepatocytes, resulted in a 5-fold potentiation of the cytotoxicity of CP. Alkaline elution analysis of cellular DNA demonstrated that the level of apparent interstrand cross-linking was 3 to 4 times higher in GSH-depleted cells than in nondepleted cells. GSH-depleted cells were, in addition, more sensitive to induction of DNA single strand breaks than nondepleted cells. Depletion of GSH content did not increase cellular sensitivity to the cytotoxicity of phosphoramide mustard.

Preincubation of K562 cells with 1 mM cysteine for 4 h resulted in an approximately 60% increase in cellular GSH content, which was accompanied by decreased sensitivity to the cytotoxicity of hepatocyte-activated CP.

Exposure of nondepleted cells to clinically relevant concentrations of hepatocyte-activated CP resulted in depletion of cellular GSH content. Replenishment of GSH content in these cells was relatively slow following CP exposure. Acrolein was highly effective at depleting cellular GSH content, whereas phosphoramide mustard had no effect on cellular GSH content. The depletion of GSH by intracellularly released acrolein may be important in the mechanism of cytotoxicity of CP.

INTRODUCTION

Glutathione is a tripeptide with a variety of functions in a number of important cellular processes (1). One role of particular importance is the protection of cellular macromolecules against reactive intermediates (2). These intermediates may be free radicals or other electrophiles, such as quinones or cations. GSH3 acts in these reactions as a nucleophile, with the formation of conjugates, or else as a reductant. Several reports have demonstrated the importance of GSH as a determinant of cellular sensitivity to anticancer drugs (3–7). Melphalan-resistant L1210 cells, shown to have elevated GSH content, have been sensitized by reduction in cellular GSH content by either nutritional deprivation of L-cysteine (3) or exposure to BSO (4), a potent inhibitor of γ-glutamylcysteine synthetase (8). Furthermore, potentiation of the cytotoxicity of Adriamycin, melphalan, and cis-platinum has been observed in both resistant and sensitive human ovarian carcinoma cell lines following GSH depletion by exposure to BSO (5). However, the effect of glutathione depletion on cellular sensitivity to activated CP has not been described. Glutathione has previously been shown to react with both acrolein (9) and 4-OH-CP (10).

In the present study, we have investigated the effect of glutathione depletion on cellular sensitivity to the cytotoxic and DNA-damaging effects of CP in a human cell line in which resistance to activated CP is primarily attributable to elevated aldehyde dehydrogenase activity, rather than to elevated glutathione content.

MATERIALS AND METHODS

Suspension culture of K562 human chronic myeloid leukemia cells, colony-forming assays in soft agarose, and activation of CP by incubation with hepatocytes prepared from phenobarbitone-pretreated rats were as described in the accompanying paper (11). All hepatocyte incubations were carried out in incubation Buffer A supplemented with 1% bovine serum albumin and 5 mM pyruvate in 25-ml Ehrlemeyer flasks. Cell-free supernatants were prepared from hepatocyte suspensions by centrifugation and added to K562 cells to a final 1/10 dilution also as described (11).

Alkaline elution of DNA and calculation of DNA cross-linking coefficient (Kc) were performed as previously described (11). All measurements of single strand breaks included a proteinase K digestion step.

Measurement of Glutathione Content of K562 Cells. The cellular content of glutathione was determined by the enzymatic recycling method based on glutathione reductase described by Griffith (12), as modified by Suzukake et al. (3). All measurements of GSH content were made on cells in early logarithmic growth, using aliquots of 10⁷ cells. dl-Buthionine-S,R-sulfoximine was obtained from Chemical Dynamics Corp., South Plainfield, NJ, initially dissolved in sterile 0.14 M NaCl/0.014 M KH₂PO₄/0.086 M KHPo₄, and further diluted in full medium prior to addition to cells. Cysteine was obtained from Sigma Chemical Co. and was dissolved in medium prior to addition to cells. In experiments to evaluate the effect of increased cellular GSH content on sensitivity to CP, K562 cells were exposed to cysteine for 4 h, centrifuged, and resuspended in fresh medium, without additional cysteine, for 30 min prior to addition of hepatocyte-activated CP.

RESULTS

Glutathione Depletion by BSO. GSH depletion was effected by exposure to 50 μM BSO for 24 h. This exposure protocol resulted in a GSH depletion of 90% with only a 10 to 12% reduction in colony-forming ability of K562 cells. Longer exposure to BSO markedly reduced survival, with only a small further decrease in GSH content. GSH depleted cells was 5.4 ± 1.1 (SD) nmol of GSH per 10⁷ cells.

Cytotoxicity of CP Metabolites in GSH-depleted Cells. The cytotoxicity of varying concentrations of hepatocyte-activated CP towards K562 cells, depleted of GSH by exposure to 50 μM BSO for 24 h, is shown in Fig. 1. GSH-depleted cells were 5 times more sensitive than cells with a normal content of GSH. In contrast, there was no increase in sensitivity of K562 cells to phosphoramide mustard following GSH depletion.

Effect of Increased Cellular GSH Content on Cytotoxicity of Hepatocyte-activated CP. Incubation of K562 cells with 1 mM cysteine (or N-acetyl cysteine) resulted in a 60% increase in...
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Fig. 1. Effect of GSH depletion on cytotoxicity of a 1-h exposure of K562 cells to hepatocyte-activated CP. •, cells exposed to 50 μM BSO for 24 h; □, non-GSH-depleted cells. Points, mean of four experiments; bars, SD.

Table 1 Effect of various agents on GSH content and sensitivity to hepatocyte-activated CP of K562 cells

<table>
<thead>
<tr>
<th>Agent</th>
<th>% of GSH</th>
<th>LD₅₀ (μM)</th>
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<tbody>
<tr>
<td>Cysteine, 1 mM</td>
<td>160</td>
<td>102</td>
</tr>
<tr>
<td>BSO, 50 μM</td>
<td>10</td>
<td>12</td>
</tr>
<tr>
<td>None</td>
<td>100</td>
<td>60</td>
</tr>
</tbody>
</table>

* GSH content of control cells was 5.4 nmol of GSH per 10⁶ cells.
* LD₅₀, concentration of hepatocyte-activated CP required to reduce colony-forming ability by 50%.

cellular GSH content after 4 h. Increased cellular GSH content was accompanied by markedly decreased sensitivity to hepatocyte-activated CP (Table 1).

Effect of GSH Depletion on CP-induced DNA Damage. K562 cells, depleted of GSH by BSO and then exposed to hepatocyte-activated CP, were more sensitive to DNA interstrand cross-linking than nondepleted cells. Cross-linking coefficient (Kᵢ), measured after 6 h of drug-free incubation, to allow maximum cross-link expression, was 3- to 4-fold higher in GSH-depleted cells exposed to 20 μM hepatocyte-activated CP for 1 h than in nondepleted cells (Fig. 2A). At higher concentrations of hepatocyte-activated CP, there was obscuring of interstrand cross-links by DNA single strand breaks, leading to a less marked increase in Kᵢ for GSH-depleted cells. In Fig. 2B, this effect is illustrated. Cells exposed to 40 μM hepatocyte-activated CP displayed only a 2-fold increase in Kᵢ due to the presence of single strand breaks.

Typical elution assays for DNA single strand breaks are shown in Fig. 2B. Single strand breaks were measured immediately following exposure to hepatocyte-activated CP, at which time the obscuring effect of interstrand cross-links was low. GSH-depleted cells showed single strand breaks following a 1-h exposure to 20 μM hepatocyte-activated CP, whereas nondepleted cells did not. Exposure for 2 h produced greater single strand breakage than a 1-h exposure in GSH-depleted cells and again did not cause single strand breaks in nondepleted cells (Fig. 2C). Measurement of single strand breaks, 6 h after a 1-h exposure to 40 μM CP, demonstrated no breaks in nondepleted cells, but marked single strand breakage in GSH-depleted cells (Fig. 2D).

Effect of Exposure to Activated CP on GSH Content of K562 Cells. Exposure of K562 cells to hepatocyte-activated CP for 1 h resulted in a concentration-dependent depletion of cellular GSH (Fig. 3). Replenishment of cellular GSH following exposure to hepatocyte-activated CP for 1 h is completed by 6 h of drug-free incubation (Fig. 4). Continuous incubation of K562 cells with hepatocyte-activated CP, without wash to remove
cells were exposed to various concentrations of acrolein for 1 h and then analyzed after various times of drug-free incubation: •, cells incubated without wash. Points, mean of three experiments; bars, SD.

GSH content of K562 cells following exposure to hepatocyte-activated CP. K562 cells were exposed to 100 µM hepatocyte-activated CP and analyzed for GSH content at various times. □, cells exposed to CP, washed after 1 h, and analyzed after various times of drug-free incubation; ■, cells incubated without wash. Points, mean of three experiments; bars, SD.

Fig. 4. GSH content of K562 cells following exposure to hepatocyte-activated CP. K562 cells were exposed to 100 µM hepatocyte-activated CP and analyzed for GSH content at various times. □, cells exposed to CP, washed after 1 h, and analyzed after various times of drug-free incubation; ■, cells incubated without wash. Points, mean of three experiments; bars, SD.

CP, demonstrated a nadir in GSH content after 2 h, followed by a slow return to control values, which was not completed until 12 h after addition of CP (Fig. 4).

Exposure to phosphoramide mustard concentrations up to 500 µmol for 4 h had no effect on K562 cell GSH content. In contrast, acrolein was a highly effective depletor of cellular GSH content (Fig. 5). Acrolein concentrations as low as 1 µmol produced measurable GSH depletion, while depletion of about 90% was observed following exposure for 1 h to 10 µM acrolein.

DISCUSSION

One of the most important of the many functions of GSH is the detoxification of reactive intermediates (2). A range of reactive chemical species, such as lipid peroxides, may be formed intracellularly either spontaneously or in reactions mediated by enzymes (2). GSH can act either as a nucleophile with the formation of conjugates or as a reducing agent, in which reaction it is oxidized to its sulfide GSSG. Depletion of the content of GSH has been shown to occur following exposure of cells to many toxic agents, and the sequence of events may ultimately result in covalent binding of reactive metabolites to critical cellular macromolecules, with consequent cytotoxicity (13, 14). Recently, evidence has been presented from several laboratories illustrating the critical importance of GSH as a determinant of cellular sensitivity to melphalan, cisplatin, and Adriamycin (4–6). Depletion of GSH has been achieved by nutritional deprivation of L-cysteine (3), by exposure to BSO (4), or by carbamoylation of glutathione reductase by nitrosoureas (7). In each case, the result of glutathione depletion is increase in cellular sensitivity to anticancer agents.

In the present study, we demonstrate a 5-fold increase in sensitivity of a human leukemia cell line to the cytotoxic effects of CP following depletion of cellular GSH content by noncytotoxic exposure to BSO. Exposure of K562 cells to 50 µM BSO for 24 h, a relatively nontoxic exposure protocol, resulted in depletion of approximately 90% of the cellular content of GSH. Subsequent exposure to hepatocyte-activated CP resulted in a 5-fold potentiation of cytotoxicity in these cells. These data establish a critical role for GSH in protection of cells against the cytotoxic effects of activated CP.

The K562 cell line is of particular interest with regard to its oxazaphosphorine sensitivity, since it has recently been shown by Hilton and Colvin (15) to have relatively high aldehyde dehydrogenase activity and to be correspondingly insensitive to activated CP, due to oxidation of 4-OH-CP/aldophosphamide into noncytotoxic derivatives. Hilton and Colvin showed that sensitivity to CP could be increased by disulfiram, an inhibitor of aldehyde dehydrogenase. The present work suggests an alternative biochemical strategy for increasing sensitivity of cells resistant to activated CP due to elevated aldehyde dehydrogenase activity.

Analysis of macromolecular DNA damage by alkaline elution demonstrated a 3- to 4-fold enhancement of interstrand cross-linking in depleted cells compared with that observed in nondepleted cells treated with the same CP concentration for 1 h (20 µM). The interstrand cross-link is thought to be the lethal lesion produced in cellular DNA following exposure to bifunctional alkylating agents (16), and the observed enhancement of cross-linking is not only a partial explanation of the increased drug sensitivity of GSH-depleted cells, but also provides important evidence that GSH can protect against the DNA cross-linking effect of activated CP. The evidence presented in this paper suggests that the potentiation of interstrand cross-linking is not attributable to decreased GSH-mediated detoxification of phosphoramide mustard in GSH-depleted cells. Thiols, including GSH, have been shown to react readily in vitro with 4-OH-CP at the 4-carbon position (10). The increased sensitivity of GSH-depleted K562 cells to activated CP which we have observed is, therefore, probably mediated through a mechanism involving 4-OH-CP. The reaction between 4-OH-CP and thiols, including GSH, with consequent loss of cytotoxic potency, has been demonstrated (10). Deactivation of 4-OH-CP by reaction with GSH prevents release of active alkylating species. Depletion of GSH should, therefore, reduce the amount of GSH available to detoxify 4-OH-CP and thereby increase the proportion that decomposes to phosphoramide mustard and acrolein.

Another interesting possibility is that acrolein, released intra-
cellularly from 4-OH-CP, may inactivate potentially important cellular macromolecules in GSH-depleted cells. Of notable importance in the case of CP is the reported inhibition of aldehyde dehydrogenase by acrolein (17). This is unlikely to be significant in cells with high GSH content, and the hepatic enzyme does not appear to be inhibited in vivo (18). However, in cells with smaller GSH content, the diminished protection against acrolein may well facilitate inhibition of aldehyde dehydrogenase with resultant increase in production of phosphoramide mustard from 4-OH-CP. Preliminary studies in our laboratory indicate that preincubation of acrolein with GSH does indeed prevent acrolein-mediated inhibition of aldehyde dehydrogenase.* In this respect, it is also noteworthy that the acrolein-mediated inhibition of NAD-linked 15-hydroxyprostaglandin dehydrogenase can be prevented, as might be expected, by prior incubation with GSH (19).

In view of our earlier observation that acrolein-induced single strand breaks are produced in nondepleted cells following exposure to activated CP (see accompanying paper), and the apparent importance of GSH in protecting cells against strand breakage, we were prompted to determine whether exposure to activated CP itself results in GSH depletion. The demonstration of concentration-dependent GSH depletion and of the relatively slow replenishment of cellular GSH following exposure to activated CP strongly suggests a role for GSH depletion in the cytotoxicity of CP. The depletion of GSH content following exposure to activated CP is probably attributable to intracellularly released acrolein. We show in this paper that acrolein is a potent depletor of GSH. In view of our demonstration that GSH provides protection against the DNA cross-linking effects of activated CP, it seems likely that the persistent depression of GSH caused by acrolein will itself facilitate increased DNA cross-linking. This may occur because of decreased GSH-mediated detoxification of 4-OH-CP and by acrolein-mediated inhibition of aldehyde dehydrogenase. The potentiation of formaldehyde-induced DNA-protein cross-links by simultaneous exposure to acrolein has been recently demonstrated (20), and this was attributed to impaired detoxification of formaldehyde, by formaldehyde dehydrogenase, caused by acrolein-induced depletion of GSH. It has been suggested that acrolein may increase the sensitivity of cells to other toxic agents by depletion of GSH and/or by inhibition of specific DNA repair enzymes (21). Although acrolein, released from 4-OH-CP, is itself unlikely to be an important directly cytotoxic metabolite of activated CP, as previous work has shown (22), the studies described in this paper indicate that it may well have a significant, albeit indirect role, via GSH depletion, in the oncoselective and tissue-selective toxicity of CP.

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REFERENCES


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