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AUGMENTATION OF ADRIAMYCIN, MELPHALAN, AND CISPLATIN CYTOTOXICITY IN DRUG-RESISTANT AND -SENSITIVE HUMAN OVARIAN CARCINOMA CELL LINES BY BUTHIONINE SULFOXIMINE MEDIATED GLUTATHIONE DEPLETION

Thomas C. Hamilton, Margaret A. Winker, Karen G. Louie, Gerald Batist, Brent C. Behrens, Takashi Tsuruo, Karen R. Grotzinger, Wilma M. McKoy, Robert C. Young and Robert F. Ozols*

Medicine Branch and Clinical Pharmacology Branch,
National Cancer Institute, Bethesda, MD 20205, U.S.A.

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The development of acquired resistance to antineoplastic drugs and the associated broad cross-resistance to other agents frequently limits the effectiveness of chemotherapy. Ling and coworkers have demonstrated that Chinese hamster ovary (CHO) cells develop the phenotype of pleiotropic drug resistance which is manifest by a decrease in drug accumulation in these cells and hence a decrease in cytotoxicity (1). The role of drug accumulation and membrane glycoproteins in the expression of primary resistance and cross-resistance in human tumors is an area of active investigation (2-4). We have developed a series of human ovarian cancer cell lines with acquired resistance to melphalan, cisplatin, or adriamycin (5). These cell lines exhibit sensitivity/resistance profiles characteristic of pleiotropic drug resistance. In addition, the melphalan and cisplatin resistant variants are also cross-resistant to irradiation (6). Both the primary resistance to melphalan and the cross-resistance to irradiation in these cell lines can be reversed by lowering glutathione (GSH) levels in the cells with buthionine sulfoximine (BSO) (6,7). In the present study, the role of GSH in the expression of sensitivity to agents other than melphalan was examined by BSO-mediated depletion of GSH. In addition, the patterns of both primary resistance and cross-resistance were compared following GSH depletion in these cell lines.

MATERIALS AND METHODS

Cell lines. Cell line A2780, an ovarian carcinoma cell line derived from an untreated patient, was provided by Dr. Stuart Aaronson (8). This cell line was made resistant *in vitro* in our laboratory by stepwise increase in the concentration of adriamycin in the growth medium from 0.1 to 2.0 uM. The adriamycin resistant variant cell line, 2780^{AD}, was then maintained in drug-free medium. Similarly, a melphalan resistant cell line (2780^{ME}) and a cisplatin resistant cell line (2780^{CP}) were developed by stepwise exposure to a final concentration of either 10 uM melphalan or 20 uM cisplatin respectively.

Assay of drug sensitivity with and without prior glutathione depletion. Cells were plated in monolayer, and 24 hr later replicate cultures received a medium change with or without 25 uM BSO (Chemical Dynamics Corp., South Plainfield, NJ). After an additional 24 hr, medium was again changed with or without fresh BSO. This BSO exposure protocol has been shown previously to reduce GSH by 70-90% with minimal toxicity (6). After 72 hr of culture, in the absence of BSO for control cells or after 48 hr of BSO-mediated GSH depletion in the alternate replicate culture, cells were harvested with trypsin and suspended at

*Address requests for reprints to this author.

a density of 10,000 cells/ml in RPMI growth medium which also contained 0.3% (w/v) agarose and chemotherapeutic agent with or without 15 uM BSO. The effect of glutathione depletion upon drug sensitivity was then determined by comparing colony formation in a double-layer agar system (2,6,9).

RESULTS AND DISCUSSION

The cell lines were most resistant to the drug that had been used in vitro to select for the resistant variant. Compared to the parent A2780 drug-sensitive cell line, 2780^{AD} was 100x more resistant to adriamycin, 2780^{ME} was 10x more resistant to melphalan, and 2780^{CP} was 14x more resistant to cisplatin. In addition, these lines expressed a pleiotropic phenotype, as the individual cell lines were partially cross-resistant to those drugs not used in the induction of primary resistance, i.e. 2780^{AD} was 3x more resistant to melphalan than A2780, 2780^{ME} was 3x more resistant to adriamycin (10), and 2780^{CP} was 4x more resistant to adriamycin and 9x more resistant to melphalan (3). While the growth rates of all the cell lines were similar, the drug-resistant variants all had increased levels of GSH. The GSH levels of 2780^{AD}, 2780^{ME} and 2780^{CP} were 2.94, 4.58, and 6.13 nmoles/10^6 cells, respectively, compared to 1.89 nmoles/10^6 cells for the drug-sensitive A2780 cell line. The BSO exposure protocol utilized reduced GSH to 0.57 nmoles/10^6 cells in A2780 and to 0.62, 0.70, and 0.71 nmoles/10^6 cells in 2780^{AD}, 2780^{ME}, and 2780^{CP} respectively. These values were found not to be significantly different from the value obtained in the parental cell line after BSO treatment (6).

Table 1 summarizes the effect of GSH depletion upon the cytotoxicities of adriamycin, melphalan, and cisplatin in A2780 and its drug-resistant variants. BSO did not potentiate significantly the cytotoxicity of 5-fluorouracil or vincristine in the drug-resistant cell lines (data not shown). As shown in Table 1, the dose of drug required to decrease clonogenic cell survival by 50% was reduced by exposure to BSO from 1.5- to 11-fold (Dose Modification Factor, DMF) when all combinations of drugs and cell lines are considered. It is apparent from the table that the greatest alteration in drug activity occurred in the relatively drug-sensitive A2780 parent cell line with its comparatively low baseline GSH concentration. Thus, BSO acts as a chemotherapeutic agent sensitizer in contrast to agents, such as verapamil, which act primarily to reverse induced resistance to vinca alkaloids and anthracyclines but generally have markedly less dose modifying capacity in sensitive cells (2,4).

Table 1. DMF values for adriamycin, melphalan, and cisplatin in human ovarian cancer cell lines following BSO-mediated GSH reduction*

	Dose modification factor		
Cell line	Adriamycin	Melphalan	Cisplatin
A2780	11.4	6.8	4.3
2780^{ME}	1.5	3.4	1.8
2780^{CP}	2.5	6.3	3.2
2780^{AD}†	1.5-5	6.0	3.8

*Results reported here are from data generated in two or more separate experiments in which each experimental point on drug dose-response curves was determined on three separate cultures and an overall mean for number of colonies present at each drug dose was calculated. DMF = (IC_{50} in the absence of BSO) ÷ (IC_{50} for drug in the presence of BSO).
†In the case of 2780^{AD}, excessive BSO toxicity necessitated use of a monolayer drug exposure procedure with subsequent cloning in agarose, as previously described (2,7). Range in DMFs derives from experiments performed with 25 or 50 uM BSO respectively.

The differences in dose modification for adriamycin, cisplatin and melphalan following exposure to BSO in 2780^{AD}, 2780^{CP} and 2780^{ME} indicate that GSH may have different roles in the expression of primary resistance to alkylating agents (cisplatin or melphalan) compared to the anthracycline antibiotic adriamycin. In 2780^{AD}, the DMF with adriamycin + BSO was 1.5-5 compared to DMFs of 3.2 to 3.4 for BSO + cisplatin in 2780^{CP} or melphalan in 2780^{ME} respectively. There were marked differences in dose modification with BSO in cell lines with primary drug resistance compared to those with cross-resistance to the same drug. Adriamycin cytotoxicity was increased markedly by BSO in A2780 (DMF=11), compared to 2780^{AD} (DMF=1.5-5). We have demonstrated previously that the resistance to adriamycin in 2780^{AD} can be partially accounted for by a decrease in net accumulation of adriamycin (2,3). In the sensitive cell line, GSH may play an important role in protecting the cell from adriamycin produced free radical induced cytotoxicity, and hence the marked effect of BSO. In contrast, an alteration in transport may be important in decreasing the cytotoxicity of adriamycin in 2780^{AD} and a reduction in GSH has a lesser role in increasing cytotoxicity. GSH depletion in cell lines that are cross-resistant to adriamycin (2780^{CP} and 2780^{ME}) resulted in a moderate increase in adriamycin cytotoxicity (DMF = 1.5 - 2.5) but did not restore sensitivity to the same level as in A2780. These results indicate that cross-resistance to adriamycin is only partially linked to GSH levels. Different effects of GSH manipulation upon melphalan cytotoxicity were observed in cell lines with primary resistance to melphalan compared to those with cross-resistance to melphalan. The DMF in 2780^{ME} was 3.4 whereas in 2780^{CP}, a cell line with a high level of cross-resistance to melphalan, the DMF was almost double (6.3). This is of particular interest since the DMF for cisplatin in this same cell line with primary induced resistance to cisplatin was 3.2. This demonstrates that for the drugs studied the quantitative relationship between GSH levels and cytotoxicity is different for primary resistance compared to cross-resistance.

We could not demonstrate previously any defects in melphalan transport in 2780^{ME} nor could we demonstrate a consistent relationship between adriamycin cytotoxicity and adriamycin accumulation in drug-sensitive and -resistant human ovarian cancer cell lines (3). Thus, in contrast to CHO cells in which cross-resistance is associated with decreased net accumulation of drug, in human ovarian cancer cells alternate mechanisms, perhaps linked to GSH levels, are important in both primary resistance and cross-resistance. The mechanisms by which GSH is associated with the expression of drug resistance in our cell lines remain to be established. The activities of GSH-linked enzymes associated with detoxification of drugs and free radicals in the drug-sensitive and -resistant cell lines are shown in Table 2. It is yet unclear as to whether any of the relatively small changes in enzyme activity observed can account for any alteration in alkylating agent metabolism or increased detoxification of adriamycin-induced free radicals. It is of interest, however, that GSH-transferase was increased in the melphalan resistant cell line, as we have demonstrated previously that, in a melphalan resistant human ovarian cancer cell line, melphalan is metabolized to a less active intermediate (7). It is also possible that GSH plays a role in the repair of damaged DNA, and studies on the effect of GSH upon formation and repair of cross-links are in progress.

The DMFs observed with BSO plus cisplatin and melphalan are of potential clinical relevance. We have demonstrated that there is a steep dose response to cisplatin in ovarian cancer patients and that prolonged survival can be achieved in some refractory ovarian

cancer patients by treating them with cisplatin at twice the dose used in their induction regimens (15). Thus, DMFs in this range may be of importance in the treatment of diseases where the dose-response relationship with alkylating agents and cisplatin is steep.

Table 2. GSH-dependent enzymes in human ovarian cancer cell lines sensitive and resistant to adriamycin, melphalan, and cisplatin *

	GSH-transferase (U/mg protein)	GSH-reductase (U/mg protein)	GSH-peroxidase (U/mg protein)
A2780	118.1 ± 15.0	38.9 ± 3.8	14.8 ± 3.6
2780^{ME}	134.9 ± 3.5	22.8 ± 5.3	5.2 ± 1.7
2780^{CP}	92.6 ± 16.8	36.6 ± 5.4	14.0 ± 2.1
2780^{AD}	147.6 ± 2.9	44.3 ± 1.1	12.9 ± 1.6

*Enzyme assays were performed on cells homogenized by hypotonic lysis in a Potter-Elvehjem homogenizer. All assays were performed in triplicate on each of at least three separate cell homogenate preparations. GSH-transferase was assayed using the method of Habig and Jakoby (11) with chlorodinitrobenzene as substrate. One unit is 1 nmole change in absorbance at 340 nm/min. GSH-reductase was measured according to Horn (12). One unit is 1 nmole NADPH oxidized/min. GSH-peroxidase was estimated as described by Paglia and Valentine (13) with H_2O_2 as substrate. One unit is 1 nmole NADPH oxidized/min. Protein was determined by the method of Lowry et al. (14) using fatty-acid free bovine serum albumin as standard.

The observation that GSH depletion does not potentiate markedly the cytotoxicity of melphalan in a CFU assay of mouse bone marrow (16) supports the possibility that BSO may have differential effects upon tumor cells compared to some non-tumor tissues. However, it is possible that GSH depletion may potentiate other toxicities of alkylating agents and preclinical toxicity studies have been initiated to determine if BSO plus an alkylating agent can be administered safely to cancer patients.

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