Role of Glutathione and Dependent Enzymes in Anthracycline-resistant HL60/AR Cells

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ABSTRACT

We studied the cellular enzymatic defenses against anthracycline-induced free radical damage in the HL60 human myelogenous leukemia cell line and in its anthracycline-resistant subline, HL60/AR. Intracellular glutathione (GSH) levels and γ-glutamyl transpeptidase activity were lower in HL60/AR than in HL60 cells. Glutathione-S-transferase (GST) and glutathione peroxidase activities were similar in both cell lines. The intracellular distribution of GSH/GST was visualized by digitized video fluorescence microscopy, utilizing the fluorescent probe monochlorobimane (MBC1), which is specifically conjugated to GSH by GST. In HL60 cells stained with the MBC1 probe, a bright diffuse cytoplasmic and nuclear fluorescence pattern was observed, whereas in HL60/AR cells, the fluorescence was mostly localized to the Golgi apparatus with a lesser component of diffuse cytoplasmic and nuclear fluorescence. Pretreatment of HL60/AR cells with buthionine sulfoximine (BSO) partially reversed resistance to daunorubicin. This effect of BSO on resistance was associated not only with the abolition of localized MBC1 fluorescence to the Golgi apparatus but also with increased intracellular accumulation and retention of daunorubicin. The results of our studies demonstrate that inhibition of GSH synthesis in HL60/AR cells results in significant sensitization to daunorubicin and suggest that changes in the intracellular distribution of GSH/GST and/or increased drug retention may be involved in mediating this effect.

INTRODUCTION

Anthracyclines are among the most important drugs in the treatment of acute leukemia and other hematological and solid malignancies (1), but their effectiveness is limited by intrinsic or acquired drug resistance in the tumor cell population (2). Anthracycline-resistant cultured cell lines have served as a model for the study of the mechanisms of cellular drug resistance. In these lines, anthracycline resistance may be associated with cross-resistance to structurally unrelated compounds (2), decreased net intracellular accumulation of drug (2, 3), increased drug degradation (4), alterations in the structure or activity of topoisomerase II (5), and changes in the pattern of intracellular drug distribution (6). In addition, GSH* and its dependent enzymes may be involved in drug resistance by affording cellular protection against free radical damage. Increased intracellular GSH levels have been shown to correlate with intrinsic anthracycline resistance (7–9), while increased activity of GSH-dependent enzymes has been associated with acquired resistance (10).

In the present study, we intended to define whether glutathione and its related cellular enzymes could be implicated in the anthracycline resistance expressed in a human leukemic cell line previously described by our group (11). The intracellular GSH levels and pertinent GSH-dependent enzymatic activities were measured in HL60 cells and in the anthracycline-resistant subline, HL60/AR. In light of our previous studies demonstrating characteristic patterns of intracellular distribution of DNR in these cells (6), the intracellular distribution pattern of MBC1—a GSH-specific fluorescent probe (12)—in both cell lines was also determined. MBC1 is less reactive than other bimanes and has been shown to react predominantly with GSH (12).

Finally, the effects of BSO, a specific inhibitor of GSH synthesis (13), on DNR accumulation, retention, and cytotoxicity were investigated in anthracycline-sensitive and -resistant cells.

MATERIALS AND METHODS

Cell Lines. The isolation and characterization of HL60 cells and its anthracycline-resistant subline HL60/AR have been previously described (11, 14). Both cell lines were maintained in suspension culture in RPMI 1640 medium, supplemented with 10% fetal calf serum.

Sterile 75-cm² tissue culture flasks containing the cells were kept at 37°C in a 5% CO₂-controlled atmosphere incubator. Cells were passed twice weekly and routinely examined for Mycoplasma contamination.

All experiments with HL60/AR cells were performed on logarithmically growing cells which had been in drug-free medium for more than a week.

Drug Sources. DNR was purchased from Ives (Wyeth) Laboratories, Philadelphia, PA. [³⁵S]DNR was obtained from the Division of Cancer Treatment, National Cancer Institute, Bethesda, MD. MBC1 was graciously provided by Dr. William W. Lee, SRI International (Menlo Park, CA). The NBD-ceramide probe was purchased from Avanti Polar Lipids (Birmingham, AL).

RPMI 1640 and fetal calf serum were purchased from Gibco Laboratories (Grand Island, NY). Bio-Rad protein determination solution was obtained from Bio-Rad (Richardon, CA). BSO, GSH, hydrogen peroxide, NADPH, CDNB, DTNB, glutathione reductase, L-γ-glutamyl-p-nitroanilide, glycyl glycine, Trizma base, Tris-HCl, Triton X-100, and sodium azide were purchased from Sigma Chemical Co. (St. Louis, MO).

Enzyme Assays. Total intracellular GSH levels were measured according to the enzyme recycling assay described by Tietze (15). GST activity was assayed as described by Habig (16), using 1 mM GSH and 1 mM 1-chloro-2,4-dinitrobenzene. GSHPx activity was assayed by the method of Paglia and Valentine (17). GGT was measured as described by Novogrodsky et al. (18). Protein determinations were carried out using the Bradford method (19).

DVFM Studies. The apparatus used for DVFM has been previously described (6, 20). Using this system, it is possible to monitor sequential
The use of a digitizer board (Data Translation, Inc.) connected to an IBM/AT computer improves image acquisition by averaging consecutive frames. In addition, colocalization studies of two or more fluorescent compounds exhibiting appropriately distinct emission wavelengths are possible by adjusting filters and threshold settings.

A stock solution of MBC1 in ethanol (0.5 mg/ml) was prepared. Prior to the addition of MBC1 to the cells, the stock solution was further diluted in RPMI 1640 medium to a final concentration of 20 to 40 μM. The final concentration of ethanol in the medium was always <5%. A stock solution of 20 mM BSO in RPMI 1640 medium was kept at −70°C. Cells were incubated for various periods in medium in the presence of BSO (1, 10, 50, and 100 μM dilutions). Control cells were incubated in appropriate vehicles not containing BSO. Subsequently, cells were exposed to MBC1 (10, 20, 40, 50, 60, 80, and 100 μM) at 25°C for 10 min. Preliminary experiments using these concentrations of MBC1 indicated that 20 to 40 μM provides adequate cell uptake for DVFM in both cell lines. After exposure to the probe, cells were pelleted and washed with Dulbecco’s PBS. They were resuspended in 10 to 20 μl of Dulbecco’s PBS, and DVFM was performed. UV optics (excitation, 395 nm; emission, 470 nm) was utilized for MBC1 and rhodamine optics for NBD-ceramide. Each experiment consisted of examination of ten or more cells in each of at least three random fields for each condition. The experiments were repeated 3 times.

Radioactively Labeled Drug Accumulation and Retention Studies. HL60 and HL60/AR cells were washed and resuspended in 2-ml tubes at a concentration of 1 million cells/ml in RPMI 1640 with 10% fetal calf serum at pH 7.4. Cell suspensions were exposed to 0.1 μM [3H]-DNR (specific activity, 30.9 mCi/mmol) in a shaking water bath at 37°C for 60 min and then washed in Dulbecco’s PBS at 2°C. The pellets were solubilized in 0.5% Triton X-100 and transferred to scintillation vials, and their radioactivity was determined. The intracellular accumulation of DNR was expressed in picomoles of drug per million cells. To study the retention of radiolabeled DNR, BSO-treated and untreated HL60 and HL60/AR cells were incubated for 60 min with 1 μM [3H]-DNR, washed, and resuspended in drug-free medium for 30 min. The amount of radioactive drug that remained associated with the cells was determined and expressed as a percentage of the initial drug accumulation.

Soft Agar Colony Growth. The effect of DNR, BSO, or a combination of these agents on the growth of HL60 and HL60/AR cells was determined utilizing a minor modification of a previously reported technique (11, 21). Cells were incubated with BSO for 14 h at 37°C in RPMI medium and subsequently exposed for 60 min to 0.01, 0.05, 0.25, 1.25, 6.25, and 20 μM DNR. After exposure to DNR, cells were washed twice in Dulbecco’s PBS, resuspended in drug-free medium, and plated in soft agar. Cells unexposed to BSO or DNR were plated as controls. After a 7-day incubation period in a 37°C, 5% CO2, fully humidified incubator, colonies consisting of 50 or more cells were counted with an inverted microscope. The IC50 was defined as that drug concentration which reduced colony formation to 50% of values obtained for untreated control cells. The DMF for DNR (DMF/DNR) was defined as the IC50/DNR without BSO pretreatment divided by the IC50/DNR after BSO pretreatment. Experiments were performed in triplicate and repeated at least 3 times for each agent tested.

Analysis of Data. In order to ascertain the significance of the differences observed between HL60 and HL60/AR cells subjected to the various experimental conditions, statistical analyses of the data were performed utilizing the Student t test. A P value greater than 0.05 was elected as the criterion for statistical significance. Values listed in the tables and figures represent the mean of at least 3 experiments ± the standard error of the mean.

RESULTS

Intracellular GSH Levels and Activities of GSH-dependent Enzymes. Shown in Table 1 are the levels of intracellular GSH and the activities of GGTP, GST, and GSHPx for HL60 and HL60/AR cells. HL60/AR cells had 1.8-fold lower intracellular glutathione levels as compared to HL60 cells. Resistant cells also had a correspondingly 2-fold lower GGTP activity. There were no significant differences in the activities of GST (toward CDNB) or GSHPx between the two cell lines. When adjusted for relative cell volume, measured by flow cytometry (data not shown), the significance of these observations was unchanged. Volume distribution curves for both cell lines were virtually superimposable. The HL60/HL60/AR mean volume ratio was 1.043.

GSH Depletion with BSO. HL60 and HL60/AR cells were incubated for 14 h in RPMI medium at 37°C in the presence or absence of various BSO concentrations. At the end of the incubation period, intracellular GSH levels were determined and expressed as a percentage of the control GSH levels. Results are presented in Table 2. Intracellular levels of GSH were reduced to a greater extent in HL60/AR cells than in HL60 cells for BSO concentrations between 1 and 10 μM. Concentrations of BSO higher than 10 μM resulted in equally marked and possibly maximal depletion of GSH in both cell lines.

DVFM Studies. In HL60 cells, the fluorescent MBC1 conjugate is distributed in a diffuse pattern in the cytoplasm and nucleus. In contrast, HL60/AR cells show less fluorescence in the cytoplasm and nucleus and localize most of the fluorescent conjugate to the Golgi apparatus (Fig. 1, A and B). Pretreatment of HL60/AR cells with BSO concentrations of 10, 50, and 100 μM abolished the localization of fluorescence in the Golgi apparatus and imparted a diffuse fluorescence distribution pattern to these cells (Fig. 1D); this effect was not observed when treatment with 1 μM BSO was utilized. In HL60 cells, the distribution of the MBC1 probe fluorescence was unaltered by BSO treatment (Fig. 1C). Colocalization studies with MBC1 and NBD-ceramide, a fluorescent probe specific for the Golgi apparatus (22), confirmed that the perinuclear localization of the MBC1 conjugate corresponded to the Golgi apparatus. Fig. 1, E and F, demonstrates that the NBD-ceramide probe identifies the Golgi apparatus in both HL60 and HL60/AR cells.

DNR Accumulation and Retention Studies. As shown in Fig. 2, treatment of HL60/AR cells with BSO for 14 h resulted in increased intracellular accumulation and retention of DNR. A single BSO concentration of 50 μM was utilized in the retention experiments. Preincubation of HL60 cells with BSO for 14 h had no effect on the intracellular accumulation or retention of DNR. Addition of 20 mM glucose to the medium did not affect the increased DNR retention observed in HL60/AR cells preincubated with BSO.
Clonogenic Survival Studies. Fig. 3 depicts clonogenic survival data for HL60 and HL60/AR cells, either untreated or sequentially exposed to BSO and/or DNR. BSO concentrations of 1 to 100 [mu]M for 14 h do not inhibit the growth of colonies in either cell line. Concentrations >100 [mu]M for this incubation period are inhibitory to both cell lines (data not shown). At the DNR concentration of 0.5 [mu]M utilized in Fig. 3, colony formation of HL60 but not of HL60/AR cells was completely inhibited. The clonogenic survival of HL60/AR cells incubated with >10 [mu]M BSO, followed by a 60-min exposure to 0.5 [mu]M DNR, was significantly reduced (P < 0.05). This effect could not be evaluated in HL60 cells, which did not form colonies at this DNR concentration. Fig. 4 depicts the pooled clonogenic survival data for HL60 and HL60/AR cells treated with 50 [mu]M BSO for 14 h, followed by exposure to varying concentrations of DNR for 60 min at 37°C. The IC50/DNR for HL60 cells...
AR cells is associated with localization of MBC1 to the Golgi

DISCUSSION

The present study suggests that resistance to DNR in HL60/AR cells is associated with localization of MBC1 to the Golgi apparatus. It also shows that inhibition of GSH synthesis in HL60/AR cells markedly increases the accumulation and retention of DNR and prevents predominant MBC1 fluorescence localization to the Golgi apparatus; these changes are associated with sensitization to DNR in HL60/AR but not in HL60 cells.

Modulation of GSH levels has been shown to alter susceptibility to anthracyclines in some studies (7, 8, 23–25), but absolute intracellular GSH levels correlate poorly with acquired anthracycline resistance (10, 25–28).

In this study, intracellular GSH levels and GGTP activity were found to be lower in the HL60/AR cells. Compared to other lines, both HL60 and HL60/AR cells are associated with a relatively high GGTP activity, perhaps reflecting an active GSH turnover (29). The greater decrease in GSH levels observed in BSO-treated HL60/AR compared to HL60 cells suggests that GSH utilization or export is increased in HL60/AR cells. An alternative explanation is that the target enzyme of BSO (γ-glutamyl cysteine synthetase) has an increased affinity for BSO in HL60/AR cells. In that regard, a recent report by Kanganis et al. provided evidence of differences in γ-glutamyl cysteine synthetase affinity for BSO between normal and neoplastic lymphocytes (30).

The activities of GST and GSHPx were identical in HL60 and HL60/AR cells. The anionic GST-α enzyme described by Batist et al. (10) in the anthracycline-resistant MCF-7 breast cancer cell line constitutes 90% of the overall GST activity in both HL60 and HL60/AR cells. The finding of equivalent GST activities in our leukemic cell lines indicates that this enzyme does not play a crucial role in anthracycline resistance in HL60/AR cells.

Our studies with DVFM visualization of the MBC1 probe suggest that, in HL60/AR cells, the localization of GSH/GST in specific intracellular sites may be a more relevant indicator of anthracycline resistance than total intracellular GSH levels or the activity of GSH-dependent enzymes. In HL60/AR cells, the fluorescence of the MBC1 probe localized more intensely in the Golgi apparatus than in other intracellular sites, whereas in HL60 cells the intracellular fluorescence was distributed diffusely.

In HL60/AR cells, BSO pretreatment promotes a loss of the bright Golgi apparatus fluorescence. It is not clear whether this occurs because of BSO-induced changes in the permeability of intracellular membranes to GSH/GST, allowing leakage between compartments, or because of site-specific GSH depletion. Nonetheless, this change in distribution pattern is associated

\[\text{DMF/DNR for HL60/AR} = \frac{0.06}{0.4} = 0.15\]

\[\text{DMF/DNR for HL60} = \frac{0.05}{0.1} = 0.5\]

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with partial reversal of DNR resistance in HL60/AR cells. Our preliminary evidence shows that re-incubation of BSO-treated HL60/AR cells (which had lost the Golgi MBC1 localization pattern) with GSH-containing medium restores the typical Golgi MBC1 fluorescence pattern. Whether drug resistance is also restored is currently being investigated.

Meredith and Reed have emphasized the importance of the mitochondrial GSH pool in the hepatocellular toxicity of Adriamycin/1,3-bis(2-chloroethyl)-1-nitrosourea (31). Mitochondrial glutathione represents 10 to 15% of the total cellular glutathione and has a turnover time several times longer than cytoplasmic GSH (32). Mitochondria lack the enzymatic machinery necessary for glutathione synthesis; therefore, mitochondrial GSH is derived entirely from the cytoplasmic compartment (33). BSO depletion of mitochondrial glutathione occurs at a slower rate than depletion of the cytoplasmic compartment (34). Although the size and importance of the mitochondrial GSH compartment in leukemic cell lines are not known, it is conceivable that differential depletion of the mitochondrial pool in HL60 and HL60/AR cells may have contributed to the reversal of drug resistance. However, the mitochondrial distribution pattern in our cell lines, as inferred from the distribution of Rhodamine 123 DVFM fluorescence, is totally dissimilar to the fluorescent pattern observed with MBC1.7 In addition, since total glutathione was reduced to less than 10% of control in both cell lines, some degree of reduction in the mitochondrial glutathione compartment in sensitive cells must also have occurred, if we extrapolate the data from hepatocytes that indicate that 10 to 15% of the total GSH is mitochondrial. If depletion of mitochondrial glutathione had a pivotal role in anthracycline resistance, one would have expected some increase in the cytotoxicity of DNR in HL60 cells as well.

The fluorescence intensity of the MBC1 conjugate is dependent upon the intracellular concentration of GSH, the activity of GST, the rate of export of the conjugate (12, 18), and degree of fluorescence quenching. Since BSO does not affect GST activity, the attenuation of Golgi fluorescence in BSO-treated HL60/AR cells suggests that the observed fluorescence represents GSH rather than GST. Further definition of this issue awaits the completion of studies of the pattern of the GSH repletion with glutathione esters (35). Unconjugated MBC1 is a lipophilic molecule, and its degree of permeability into the various cell compartments may vary according to the composition of intracellular membranes. It is possible that differences in membrane composition between HL60 and HL60/AR cells may partially account for the localization of the probe to different sites in the two cell lines.

A potential role for GSH/GST localization in the Golgi apparatus of HL60/AR cells in anthracyline resistance is suggested by DVFM studies performed in our laboratory, which demonstrate that the intracellular itinerary of DNR is markedly different in HL60 and HL60/AR cells.7 In HL60 cells, DNR diffuses freely into cytosolic and nuclear compartments at neutral pH. In HL60/AR cells, perhaps due to acquired changes in membrane glycoproteins, DNR uptake occurs in successive phases. Initially, DNR is seen in the plasma membrane, followed by localization of the drug into the Golgi area, and then in "punctate-appearing" intracellular compartments, from which the drug may be exocytosed. Our results allow the hypothesis that the presence of GSH or GST in the Golgi apparatus of resistant cells mitigates free radical damage to membranes by activated DNR "in transit," thereby preventing leakage of the drug into the cytosol. Once in the cytosol, the DNR could gain access to target macromolecules and the nucleus. Further support for this hypothesis is derived from our preliminary observation that BSO-treated HL60/AR cells exhibit increased fluorescence of DNR in the cytosolic/nuclear compartment.8

A recent article by Kramer et al. (25) demonstrated that, in the MCF7/Adr and P388/Adr cell lines, glutathione depletion plus treatment with verapamil totally reversed anthracycline resistance, whereas only partial reversal was observed when each treatment was used alone. Treatment with buthionine sulfoximine did not increase the accumulation of anthracyclines in the MCF7/Adr line. There are major differences between the cell lines studied in that paper and HL60/AR cells. Although HL60/AR cells exhibit the pharmacokinetic characteristics of classic multidrug resistance (11), they do not overexpress the P-glycoprotein;9 the M, 160,000 and 110,000 glycoproteins originally described in HL60/AR cells appear to represent hypoglycosylated forms of glycoproteins that are present in both cell lines (36). In addition, HL60/AR cells do not demonstrate increased GST activity (such as MCF7/Adr) or increased GSH peroxidase activity (such as P388/Adr). In HL60/AR cells, unlike in MCF7/Adr cells, BSO treatment markedly increased drug accumulation and retention, while partially reversing drug resistance. To our knowledge, this constitutes the first evidence of a role for glutathione in the accumulation and retention of anthracyclines.

The mechanism by which pretreatment with BSO increases DNR accumulation and retention in HL60/AR cells is unclear, but several plausible hypotheses exist. Treatment of HL60/AR cells with metabolic inhibitors increases DNR retention; this effect is reversed by the addition of glucose.7 In our studies, however, addition of glucose does not reverse the BSO-mediated increase in DNR retention. Although GSH levels in HL60 cells were similarly decreased by BSO treatment, an effect on accumulation or retention could not be demonstrated. This is likely due to the fact that maximal DNR retention in viable HL60 and HL60/AR cells is within the range of 65 to 75%; higher retention values are observed only in nonviable cells.

We have found that baseline lipid peroxidation is 3 times lower in HL60/AR than in HL60 cells,4 as measured by the thiobarbituric acid assay. Lowering of GSH levels could also affect membrane permeability by increasing the susceptibility of membrane components to oxidative damage. The effect of BSO on lipid peroxidation in our lines is currently under investigation.

Another possibility is that GSH depletion renders cell membranes more permeable to daunorubicin by altering their lipid composition. Synthesis of certain leukotrienes from membrane arachidonate has been shown to be GSH dependent (37, 38).

Changes in protein thiols (39) or in microtubular function (40, 41) caused by GSH depletion may interfere with the accumulation and intracellular handling of DNR in HL60/AR cells.

It is also possible that glutathione depletion changes the cell membrane permeability properties. Additionally, GSH depletion may alter the rate at which drug enters the cell. This is critical, since the accumulation of DNR may be limited by the size of the drug and the capacity of the cell to incorporate the drug into its membrane.

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8 A. A. Hindenburg and J. Lutzky, unpublished observations.

amount of daunorubicin that becomes covalently bound to cellular macromolecules. Covalent binding between anthracyclines and phospholipids, proteins, and nucleic acids has been demonstrated (42-44). Covalent protein binding in \textit{in vitro} microsomal systems has been shown to be inhibited by glutathione and other sulfhydryl compounds (42).

In summary, our findings provide evidence that, in the HL60/AR cell line, GSH or GST is preferentially localized to an intracellular site involved in DNR traffic. Whether the effect of glutathione depletion on the distribution of GSH/GST is related to the increased drug accumulation and retention caused by BSO treatment, or whether these are two separate consequences of glutathione depletion remains the subject of further investigation. Pharmacological inhibition of GSH synthesis partially reverses anthracycline resistance in HL60/AR cells. Absolute GSH levels or GST activity does not appear to play a critical role in the anthracycline resistance exhibited by these cells, as reported in other cell lines. It is possible that following GSH depletion, leakage of DNR from the Golgi apparatus into the cytosol would allow the drug to attack other targets such as the mitochondria (which may be more susceptible to damage after glutathione depletion), the nuclear membrane, and the nucleus.

The data presented in this paper support the concept that multiple mechanisms may be involved in the development of acquired anthracycline resistance to anthracycline drugs in experimental cell lines. Localization of GSH/GST to specific subcellular compartments could contribute to another such mechanism in HL60/AR cells.

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