Reduced Levels of Drug-induced DNA Cross-Linking in Nitrogen Mustard-resistant Chinese Hamster Ovary Cells Expressing Elevated Glutathione S-Transferase Activity

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Abstract

We have reported previously (C. N. Robson et al., Cancer Res., 46: 6290–6294, 1986) the isolation of a Chinese hamster ovary cell line, designated CHO-Chl, that exhibits resistance to bifunctional nitrogen mustards while maintaining the normal parental level of sensitivity to several other alkylating agents. We have compared the rate of formation and repair of DNA cross-links induced by mechlorethamine in CHO-Chl and parental CHO-K1 cells, both in intact cells and in isolated nuclei. Equimolar doses of mechlorethamine induce significantly fewer DNA interstrand cross-links in CHO-Chl cells than in CHO-K1 cells, but levels of DNA-protein adducts are approximately equivalent in these two lines. There is a correlation between the relative resistance of CHO-Chl cells to mechlorethamine (34-fold) and the amount of drug required to induce approximately equal numbers of DNA interstrand cross-links in the two cell lines. This strongly implicates DNA-DNA adducts in the cytotoxic action of mechlorethamine. DNA cross-linking studies on isolated nuclei reveal only minor differences between the two lines even with identical drug treatments. The rate of cross-link repair is comparable in the two cell lines. These results, taken together with our earlier observations that the rate of drug accumulation is identical in these two lines, suggest that enhanced cytoplasmic drug detoxification is the underlying resistance mechanism in CHO-Chl cells. We have measured cellular glutathione S-transferase activity, using both the general substrate 1-chloro-2,4-dinitrobenzene and substrates with some specificity for the different classes of transferase isoenzymes. Total enzyme activity (as measured with 1-chloro-2,4-dinitrobenzene) is elevated 3-fold in the resistant cells. A 2- and 5-fold increase, respectively, in activity against ethacrynic acid and cumene hydroperoxide is detectable in CHO-Chl cells. This elevation in catalytic activity in the resistant cells is reflected in higher levels of both the Yr- and Yc-type transferase subunits.

Introduction

Alkylating agents represent an important class of antitumor drugs. However, as with several other groups of clinically useful cytotoxic agents, their effectiveness is limited by the emergence of subpopulations of resistant tumor cells. Several mechanisms of resistance to alkylating agents in human and rodent cell lines have been reported, including reduced membrane permeability, increased efficiency of DNA repair, and enhanced drug detoxification (1–10). Indeed, the mechanism responsible for nitrogen mustard resistance is frequently multifactorial. For example, a melphalan-resistant CHO3 cell line was reported to have an increased level of sulhydryl groups, enhanced drug efflux, and a decrease in drug-induced DNA cross-linking (1).

Several alkylating agents are sufficiently electrophilic to react directly with the major cellular non-protein thiol-containing compound, GSH. However, mammalian cells have evolved a supergene family of enzymes, the GSTs, which catalyze this conjugation reaction (11). The GSTs are believed to be responsible for catalyzing the conjugation of GSH to electrophilic metabolites formed from nitrogen mustards (12, 13). The resultant decrease in the steady state level of reactive drug metabolites spares critical intracellular targets such as DNA from levels of alkylation that cannot be repaired effectively.

We reported recently the isolation of a CHO cell line (CHO-Chl) with acquired resistance to bifunctional nitrogen mustards (14). This subline was isolated on the basis of chronic exposure to chlorambucil and exhibits resistance not only to chlorambucil (24-fold) but also to melphalan (14-fold) and mechlorethamine (34-fold). In contrast to the chlorambucil-resistant Walker rat carcinoma (WR) cell line (15), CHO-Chl cells are not cross-resistant to other bifunctional alkylating agents, such as mitomycin C, cis-diaminedichloroplatinum(II) or cyclophosphamide, nor are they collagenously sensitive to nitrosoureas.

We have investigated the mechanism of resistance to nitrogen mustards in CHO-Chl cells. Evidence is presented that the principal alteration found in the resistant cells following exposure to drug is a reduction in the extent of damage to DNA. This reduction is accompanied by an apparent increased rate of drug detoxification that is a consequence of an elevation in GST activity coupled with a small increase in cellular GSH content.

Materials and Methods

Cell Culture and Media. Cells were routinely maintained in Ham's F-10 medium supplemented with 5% fetal calf serum, 5% newborn calf serum, glutamine (3 mM), and antibiotics (penicillin, 100 units/ml; streptomycin, 100 µg/ml; and nystatin, 50 units/ml). Cells were grown as monolayers at 37°C in a humidified atmosphere containing 5% CO2. Cell Labeling and Alkaline Elution. Cellular DNA was labeled by incubating cells with [2-14C]thymidine (0.02 µCi/ml) for 48 h. Following growth in isotope-free medium for 16 h, cells were exposed to mechlorethamine for 1 h and, where indicated, then incubated in drug-free medium to follow the time course of DNA repair. DNA-protein and DNA-DNA interstrand cross-links were assayed by the method of alkaline elution, essentially as described by Kohn et al. (16). Briefly, approximately 5 x 105 cells (which were X-irradiated on ice prior to elution) were impinged onto polyvinyl chloride filters (2-µm pores) lysed with 40 mM EDTA-2 M NaCl-0.2% sodium dodecyl sarcosine (pH 10.0), and, where indicated, exposed to 0.5 mg/ml proteinase K for 1 h. The DNA was eluted from the filters at a rate of 0.035 µl/min using a solution of 20 mM EDTA (free acid) adjusted to pH 12.1 with tetrapropylammonium hydroxide (for the measurement of total cross-links). For DNA-DNA cross-links, the elution buffer contained 0.1% sodium dodecyl sulfate. Fractions were collected at 90-min intervals over 15 h.

The use of a Watson-Marlow 202U/AA 16 channel constant speed peristaltic pump gave highly reproducible flow rates in all channels.

Nuclei were prepared by the method of Glisson et al. (17). Isolated...
nuclei were exposed to drug in PBS at 37°C and then harvested and resuspended in ice-cold PBS prior to X-irradiation (600 rads).

Cross-linking factors are defined as the ratio of the log fraction DNA retained after 9 h in the irradiated control and the log fraction DNA retained in the sample treated with both mechlorethamine and radiation, as described by Ross et al. (18).

Glutathione S-Transferase Assays. GST activity towards the substrates 1-chloro-2,4-dinitrobenzene and ethacrynic acid was measured by the method of Habig and Jakoby (19). Peroxidase activity towards cumene hydroperoxide was carried out as described by Stockman et al. (20). Cell suspensions were sonicated and spun for 5 min at 13,000 rpm in an Eppendorf microfuge, and the supernatants were assayed.

Dot Blots. This was performed to measure the relative levels of the different classes of GSTs in the 2 cell lines. The antisera used were raised in rabbits against the human proteins GST λ, GST μ, and GST B,B, which are members of the families referred to as "acidic," "neutral," and "basic" class GSTs, respectively. The specificities of these antisera towards purified rat, mouse, guinea pig, and hamster GSTs have been described elsewhere (21, 22).

Cytosolic proteins from CHO-Chl' and CHO-K1 cells were dotted onto nitrocellulose filters, washed for two 10-min periods in TBST, and blocked for 1 h with 3% (w/v) bovine serum albumin in TBST. Two 10-min washes with TBST were carried out, after which filters were incubated for 1 h with a specific human antibody to each of the three GST isoenzyme classes. Following four 15-min washes with TBST, the filters were incubated for 1 h with anti-rabbit IgG conjugated to horseradish peroxidase antibody. The filters were washed and dots were visualized either using 4-chloro-1-naphthol or by autoradiography after labeling with 0.19 MBq 125I-protein A.

Immunocytochemistry. Immunocytochemical staining of air-dried cytoscintifuge preparations of intact cells was performed using an adaptation of the method of Swirsky et al. (23). Briefly, cells were fixed for 2 min in methanol:acetone (1:1, v/v) and then washed with PBS. A 100-μl aliquot of primary antibody (raised against human acidic, neutral, or basic forms of GST) was added to each slide and incubated at room temperature for 45 min. Control slides were incubated with Earle's balanced salt solution. Following incubation, slides were washed with PBS and immersed for 10 min in 100 ml of methanol:7 ml of 30% (v/v) hydrogen peroxide. After further washing with PBS, 100 μl of secondary antibody (swine anti-rabbit antibody conjugated with horseradish peroxidase diluted 1:40 in Earle's balanced salt solution containing 5% normal inactivated human serum) were added to each slide and incubated for 45 min. Further washing with PBS was followed by development of peroxidase for 10 min in a mixture of 50 mg of 3,3'-diaminobenzidine in 100 ml of PBS and 20 μl of H2O2. Slides were then washed in distilled water and counterstained with hematoxylin.

RESULTS

DNA Cross-Linking in Resistant and Parental CHO Cells. DNA cross-linking induced by mechlorethamine was measured in CHO-Chl' and CHO-K1 cells using alkaline elution (16). Representative elution profiles for CHO-K1 cells following a 1-h exposure to different concentrations of drug are shown in Fig. 1. The effect of increasing doses of mechlorethamine is to reduce the rate of DNA elution, reflecting the formation of DNA cross-links.

The level of both DNA interstrand and DNA-protein cross-links was measured in resistant and parental cells. DNA interstrand cross-linking frequencies were determined by including proteinase K in the elution protocol, in order to eliminate DNA-protein adducts. The level of DNA interstrand cross-linking induced by equimolar doses of mechlorethamine is very significantly higher (P < 0.001) in CHO-K1 cells than in CHO-Chl' cells (Fig. 2). CHO-Chl' cells have previously been shown in clonogenic survival assays to be 34-fold resistant to mechlorethamine (14). Based on this degree of resistance, it can be seen (Fig. 2) that approximately equitoxic doses of drug induce the same level of DNA-DNA adducts in the 2 cell lines. For example, mechlorethamine doses of 0.12 and 4 μg/ml, respectively, produce a cross-linking factor of 2 in parental and resistant cells.

Levels of DNA-protein cross-links were assessed by calculating the difference between total cross-links (absence of proteinase K) and DNA interstrand cross-links. Both lines accumulate similar numbers of DNA-protein cross-links, although the resistant cells show a small but consistently increased level of these adducts (data not shown). Thus, the strikingly higher level of DNA cross-links seen in CHO-K1 cells appears to be solely a consequence of an increase in the number of DNA-DNA adducts.

Repair of DNA Cross-Links. To assess the DNA repair proficiency of the two lines, cells were exposed for 1 h to a dose of mechlorethamine that induced the same level of DNA interstrand cross-links, before being washed and returned to drug-free medium. At intervals, samples were taken and the degree of cross-linking was determined. Fig. 3 shows that the rate of cross-link repair is not significantly different in parental and resistant cells.

Cross-Linking in Isolated Nuclei. To determine whether the low level of drug-induced DNA damage in CHO-Chl' cells is a consequence of a reduced level of active drug being accumulated
NITROGEN MUSTARD-RESISTANT CHO CELLS

Fig. 3. Kinetics of repair of DNA interstrand cross-links induced by mechlorethamine in CHO-K1 (●) and CHO-Chl' (○) cells. Points, means of 2 independent determinations.

Mechlorethamine

Time of repair (hours)

0  4  8  12  16  20  24

Cross-linking factor

Fig. 4. Levels of DNA interstrand cross-links induced in nuclei isolated from either CHO-K1 cells (●) or CHO-Chl' cells (○), as a function of mechlorethamine concentration. Points, means of at least 3 independent experiments; bars, SE.

in the nucleus of whole cells, cross-linking measurements were repeated using isolated nuclei. In this case, far lower concentrations of mechlorethamine were required to give measurable levels of cross-links. Although slightly higher numbers of cross-links were induced in nuclei isolated from parental CHO-K1 cells than those from the resistant cells (Fig. 4), the difference is not significant ($P > 0.05$) and far less striking than that seen in similar studies with intact cells.

These results suggest either that lower levels of drug are accumulated intracellularly by CHO-Chl' cells or that drug detoxification in the cell cytoplasm occurs more efficiently. The former possibility can be discounted inasmuch as we have previously shown that the rate of drug accumulation is not significantly different in CHO-Chl' cells (14). Thus, drug detoxification would appear to be the most likely mechanism of resistance.

The GST family of isoenzymes has been reported to be involved in the detoxification of a variety of xenobiotics, including alkylating agents, via conjugation with reduced GSH (11, 12). The levels of these enzymes in the two cell lines were therefore measured.

Measurements of GST Activity. Initially, transferase activity using 1-chloro-2,4-dinitrobenzene as substrate was measured in parental and resistant cells. Activity against 1-chloro-2,4-dinitrobenzene reflects overall levels of the different transferase isoenzymes. Fig. 5 shows that CHO-Chl' cells have a 3-fold higher total GST activity. In order to obtain an indication of the enzyme subgroups that are overexpressed, assays were performed using substrates that show a degree of specificity for the neutral ($\mu$, $\gamma_b\gamma_b$), acidic ($\lambda$, $\gamma_b\beta$), or basic ($\epsilon$, $\beta_b\beta$) isoenzyme forms. No activity against styrene oxide (for the neutral isoenzymes) was detected. However, a significant elevation in both cumene hydroperoxide (5-fold) and ethacrynic acid (2-fold) activities was seen. These activities are indicative of the basic and acidic GST isoenzymes, respectively, although more than 1 class of GST may show significant activity with these substrates.

Immunological Measurements of GST Levels. To confirm that the increased catalytic activity against ethacrynic acid and cumene hydroperoxide was reflected in increased GST levels, immunological measurements of GST levels were performed. Higher levels of cross-reaction with the $\gamma_f$-specific antisera (acidic) and, more particularly, the $\gamma_s$-specific antisera (basic)
Fig. 7. Immunocytochemical analysis of fixed CHO-K1 (A, C, E, and G) and CHO-Chl' (B, D, F, and H) cells. Cells were exposed to either no antibody (A and B) or antibodies specific for the human basic (C and D), neutral (E and F), or acidic (G and H) isoenzyme forms. Treatment conditions are outlined in "Materials and Methods." The intensity of staining is an indication of the intracellular GST concentration.

are seen in CHO-Chl' cells, as revealed by either dot blotting (Fig. 6) or immunocytochemical analysis (Fig. 7).

DISCUSSION

We have studied the level of DNA damage induced by mechlorethamine in cell lines resistant or sensitive to bifunctional nitrogen mustards. The striking difference between cross-link levels seen in intact cells, but not in isolated nuclei, is indicative of reduced intranuclear accumulation of active drug in the resistant cells. Because total drug uptake into whole cells is apparently unchanged in CHO-Chl' cells (14) it appears likely that an increase in cytoplasmic drug detoxification is the underlying mechanism of drug resistance in these cells.

Intracellular glutathione has a major role in the detoxification of reactive metabolic intermediates. Although spontaneous reactions with electrophilic molecules can occur readily in vitro, mammalian cells contain a variety of GST isoenzymes that appear to catalyze these reactions in vivo. A large number of distinct GST isoenzymes have been purified from human, mouse, and rat cells, and in each species the different forms have been shown to be members of three separate families. In humans, mice and rats, GSTs that are members of the same family display immunochemical cross-reactivity, exhibit similar catalytic properties, and possess sequence homology (11, 24). The GSTs in the Chinese hamster have not been well characterized (25, 26), but our data indicate that the three families are represented in this species.

Changes in the intracellular level of GST activity have been reported previously in alkylating agent-resistant cell lines. For
example, the chlorambucil-resistant Walker carcinoma WR cell line has 2-fold higher GST activity than the parental WS line, although in 2 cases (ethacrynic acid and trans-4-phenyl-3-buten-2-one), subunit specific activities are higher in the parental cells (7). In a Yoshida cell line resistant to cyclophosphamide, an approximately 6-fold higher GST activity was seen compared to the sensitive parental line (4). An in vivo model for drug resistance in ovarian cancer also implicates elevated GSH and GST levels (27). In our CHO-Chl' cells, GST activity is elevated 3-fold, which appears to be a consequence of an increase in the level of 2 enzyme forms. Whether the M, 25,000 protein previously reported to be overproduced in CHO-Chl' cells (14) is a GST subunit and associated with the observed increase in GST activity in these cells is currently under investigation. Although CHO-Chl' and the Walker WR cells show comparable levels of resistance to nitrogen mustards (14, 15), CHO-Chl' cells differ from WR cells in being sensitive to other classes of bifunctional alkylators, such as cis-diaminedichloroplatinum(II) and mitomycin C. The narrow range of drugs to which CHO-Chl' are resistant indicates that this line may represent a good model for studying the substrate specificities of the different transferase isoenzymes and their cellular function. This may be particularly relevant because of the apparent lack of accompanying changes in the resistant cells, such as membrane permeability.

Analysis of DNA damage showed a dramatic reduction in the level of DNA cross-linking induced by melphalan in CHO-Chl' cells. This difference was almost entirely due to the formation of DNA-DNA cross-links, not DNA-protein adducts. Indeed, it appears that slightly more DNA-protein cross-links were seen in the resistant than the parental cells at equimolar drug doses. If the GSTs were inactivating nitrogen mustard-derived nucleophiles, it may be expected that monoadducts as well as cross-links would be prevented or quenched, and hence the level of DNA protein cross-links would also be reduced in CHO-Chl' cells. It is possible that GSTs are able to inactivate monoadducts already bound to DNA by conjugation with GSH or that the GSH-conjugated nitrogen mustard derivatives are still capable of monovalent attack and that this could produce equivalent levels of DNA protein cross-links in the two lines. Further experiments are required to address these possibilities. There is evidence that GSH-conjugated nitroimidazole derivatives can still radiosensitize (28) and certain GSTs are bound to interchromatic regions of the cell nucleus (nonhistone protein BA is a Yb,Yb glutathione transferase) (29).

Our results support the contention that the principal lethal lesion generated by nitrogen mustards is the DNA interstrand cross-link. There appears to be no correlation between the formation of DNA-protein adducts and the cytotoxic effects of melphalan. The apparent relationship between increased drug detoxification and a reduction in the level of DNA cross-linking provides additional evidence that GST can directly protect cells against the lethal effects of alkylating agents. The minor difference in cross-link levels seen in experiments with isolated nuclei may reflect the presence of low levels of GST in the nuclei of normal cells or of contamination by cytoplasmic components of the prepared nuclei.

Our previous observation that the steady state intracellular GSH content of CHO-Chl' cells is elevated approximately 2-fold (14) indicates that acquired drug resistance in these cells is presumably mediated both by this increase in acceptor sulfhydryl concentration and the overexpression of specific GST subunits.

The rate of repair of DNA-DNA adducts is apparently unchanged in the resistant cells. In contrast to that seen previously in a melphalan-resistant CHO cell line (1), the rate of repair of mechlorethamine-induced cross-links is relatively rapid in our cells and cross-link numbers did not reach a peak 3 h after the removal of drug. It is difficult to make definitive statements concerning the similarity in DNA repair capacity of 2 cell lines, particularly when the levels of cross-links measured represent a balance between continuing formation and loss by repair. However, it would appear that there are no gross changes in DNA repair capacity in CHO-Chl' cells.

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