Cancer Chemother Pharmacol (2000) 46: 255–262 © Springer-Verlag 2000

ORIGINAL ARTICLE

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Decreased cisplatin/DNA adduct formation is associated with cisplatin resistance in human head and neck cancer cell lines

Received: 9 December 1999 / Accepted: 10 May 2000

Abstract *Purpose*: To evaluate the correlation between cisplatin sensitivity, intracellular glutathione, and platinum/DNA adduct formation (measured by atomic absorption spectroscopy) in a series of seven head and neck cancer cell lines, and to evaluate the effect of biochemical modulation of glutathione on platinum/DNA adduct formation and repair. *Methods*: Cisplatin/DNA adducts were measured by atomic absorption spectroscopy. Glutathione content was measured by enzymatic assay and was modulated with buthionine sulfoximine. Apoptosis was measured by double-labeled flow cytometry. *Results*: Intracellular glutathione concentration was strongly correlated with cisplatin resistance ($P = 0.002$, $R^2 = 0.7$). There was also a statistically significant inverse correlation between cisplatin/DNA adduct formation and the IC_{50} for cisplatin in these cell lines. ($P = 0.0004$, $R^2 = 0.67$). In addition, resistant cells were able to repair approximately 70% of cisplatin/DNA adducts at 24 h, while sensitive cells repaired less than 28% of adducts in the same period. However, despite the positive correlation between cellular glutathione and cisplatin resistance, there was no direct correlation between intracellular glutathione concentration and platinum/DNA adduct formation. Further, depletion of intracellular glutathione by buthionine sulfoximine did not dramatically alter formation of cisplatin/DNA adducts even though it resulted in marked increase in cisplatin cytotoxicity and was associated with increased apoptosis. *Conclusions*: These results suggest that glutathione has multiple effects not directly related to formation of cisplatin/DNA adducts, but may also be an important determinant of the cell's ability to repair cisplatin-induced DNA damage and resist apoptosis.

Key words Head and neck cancer · Cisplatin · Glutathione · Apoptosis

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Introduction

Cisplatin-based chemotherapy is widely used in the management of locally advanced or recurrent squamous carcinoma of the head and neck. Originally reserved for patients with relapsed disease, cisplatin-based drug regimens have been used increasingly in combination with drugs such as 5-fluorouracil or the taxanes (paclitaxel and docetaxel) to preserve organ function either in sequence with radiation treatment or given concomitantly [1, 23].

While cisplatin-based chemotherapy is highly effective in previously untreated patients, with partial response rates in excess of 90% and complete responses in excess of 50% [14], the same cisplatin-based chemotherapy is dramatically less effective in patients treated for relapsed disease. In this group, clinical responses occur in less than one-third of patients and complete responses are extremely unusual [9].

Cisplatin is believed to induce tumor cell death by the formation of platinum/DNA adducts which ultimately inhibit DNA replication [11]. The ability of the tumor cell to resist adduct formation at a clinically feasible dose of cisplatin as well as the ability of the tumor to repair platinum/DNA damage once it has occurred are felt to be the key determinants of response to cisplatin-based chemotherapy in these tumors [10].

A number of cellular defense mechanisms are important mediators of cisplatin resistance in clinical tumors. Glutathione, a ubiquitous tripeptide, is an important host defense molecule against a range of toxins including

metal compounds such as cisplatin [6]. Various lines of experimental evidence have led to the hypothesis that glutathione within the cell cytoplasm can bind metal compounds such as cisplatin and prevent DNA adduct formation.

The conjugation of electrophile compounds to glutathione can be catalytically enhanced by a series of enzymes known collectively as the glutathione *S*-transferases (GSHs) [3, 12]. In head and neck cancers, the most widely expressed of these is glutathione *S*-transferase π. Once glutathione has conjugated cisplatin or a similar toxic compound, the conjugate is removed from the cell by an energy dependent pump mechanism. Several cell surface proteins including the multidrug resistance-associated protein have been described which may serve as conduits by which cisplatin/GSH adducts are removed from the cell [29].

Previous studies have demonstrated that increased intracellular glutathione is associated with cisplatin resistance in head and neck cancer cell lines [20]. Additionally, expression of glutathione *S*-transferase π has also been associated with cisplatin resistance in a number of malignancies, including head and neck [21], ovarian, and lung [2, 13] tumors. However, there is little direct evidence examining the role of glutathione in preventing cisplatin/DNA adduct formation. Glutathione has also been felt to participate in cellular DNA repair mechanisms as well as pathways leading to apoptosis. Recent studies have demonstrated that expression of gene products critical to the regulation of apoptosis including Bcl-2 and Bax may be significantly regulated by glutathione [4, 5, 30].

In the present study, we have evaluated cisplatin/DNA adduct formation in head and neck tumor cell lines directly through the use of atomic absorption spectroscopy.

Materials and methods

Cell lines and cell culture

Seven cell lines were used for this investigation. All were squamous cell carcinomas established in other laboratories from squamous tumors of the upper aerodigestive tract. Cell lines UM-SCC-22A, UM-SCC-17B, and UM-SCC-38 were isolated by Dr. Thomas Carey at the University of Michigan Cancer Center. Lines PCI-13 and PCI-51 were provided by Dr. Theresa Whiteside of the University of Pittsburgh. Lines SCC-25 and SCC-25CP were initially described by Teicher et al. [24] and were provided by Dr. J. Lazo, University of Pittsburgh. SCC-25CP is a cisplatin-resistant line that was derived from SCC-25 after repeated exposure to cisplatin in culture. All cell lines were routinely cultured in IMEM (Biofluids, Rockville, Md., USA) with 10% fetal calf serum.

Cytotoxicity assay

Tetrazolium XTT assay was used to determine the relative sensitivities of seven human head and neck cancer cell lines to cisplatin. The seven cell lines were plated in 96-well plates at 5000 cells per well in 100 µl medium. All cell lines were plated in triplicate. Following overnight incubation, cells were exposed to various concentrations of drug, which was added in 100-µl volumes. After 72 h incubation, 50 µl of a mixture of XTT (1 mg/ml in IMEM) and phenazine methosulfate (15.3 µg/ml) was added per well. The optical densities of wells that had been treated with cisplatin were then compared with optical densities of control wells that had not been treated with cisplatin. The IC_{50} was defined as the concentration of cisplatin causing a 50% decrease in control absorbance. To test the effect of glutathione modulation on cisplatin sensitivity, UM-SCC-17B and SCC25CP cell lines were treated with buthionine sulfoximine (BSO) at a concentration of 1 mM for 24 h to deplete glutathione or *N*-acetyl-L-cysteine (NAC) at a concentration of 5 mM for 24 h to increase glutathione content. After pretreatment with BSO or NAC, cisplatin was added to the sensitive cell line (UM-SCC-17B 0.8 µM) and to the resistant cell line (SCC25CP-8 µM). The remainder of the assay was as described above.

GSH measurement

Total intracellular GSH was measured in seven cell lines based on the enzymatic method of Tietze [25]. Twenty-five micrograms of protein from cell lysate for each cell line was used in the assay as described. Briefly, cells were plated in a T-75 flask. When they had reached 80% confluence, cells were removed with trypsin, washed twice in ice-cold PBS and resuspended in normal saline. The cells were frozen and thawed 3 times and then sonicated. The protein concentration of the cell lysate was determined by the modified Bradford method (BioRad, Hercules, Calif., USA). Cell lysate (25 µg) was then used for GSH determination. A standard curve was run for each assay using purified GSH (Sigma, St. Louis, Mo., USA).

DNA platination

To measure cisplatin/DNA adduct formation, exponentially growing cells in triplicate 150-cm^2 dishes were incubated with 50 µM cisplatin for 1 h at 37 °C. After 1 h exposure, medium was removed, and cells were harvested by trypsinization and washed twice with ice-cold PBS. Cell pellets were incubated for 5 h at 37 °C in lysis buffer (10 mM TRIS-HCl, pH 8.0) containing 0.1 M NaCl, 0.1 mM EDTA, 0.5% (w/v)SDS, and 20 µg/ml RNase. Proteinase K (100 µg/ml) was then added, and lysates were incubated overnight at 50 °C. DNA was isolated by the phenol/chloroform method and then dissolved in 500 µl Tris/EDTA. DNA concentration was measured by absorbance at 260 nm [16]. Platinum content was measured by atomic absorption spectroscopy. The DNA platination levels were expressed as pg of platinum per µg of DNA. All platination measurements were made in triplicate. Standard error for a triplicate measurement was usually less than 10%. All experiments were repeated three times.

Atomic absorption spectroscopy

All samples and standards were analyzed on a Zeeman 5000 atomic absorption spectrometer (Perkin-Elmer, Norwalk, Conn., USA) fitted with an HGA500 graphite furnace and autosampler AS-40. The apparatus was equipped with a platinum hollow cathode lamp (Buck Scientific, East Norwalk, Conn., USA). The software instrument controller program was AutoAA (Analytical Instrument Services, Mt. Airy, Md., USA). Standards were prepared from a commercial atomic absorption platinum standard (1000 µg/ml in 5% HCl; Sigma). A stock platinum solution of 1 µg/ml was prepared fresh each week and standards were serially diluted to 100, 50, 25, 15, 10, 7.5, and 0 ng/ml. All standards and samples were run in triplicate. Twenty-five microliters of sample or standard were injected onto the graphite tube (Perkin-Elmer). Triplicate measurements were made of each sample. All samples and standards were observed at wavelength 265.9 nm with slit width 0.7 nm and lamp current 20 mA.

DNA repair

To determine repair of platinum/DNA adducts, a cisplatin-sensitive cell line (UM-SCC-17B) and resistant cell line (SCC25CP) were selected. Each cell line was incubated with 50 μM cisplatin for 1 h at 37 °C. Cells were then washed twice in PBS, and either harvested immediately or cultured in drug-free medium and harvested at 1, 6, and 24 h. DNA was isolated and analyzed for platinum content as above.

BSO treatment

To investigate the relationship between DNA platination and GSH levels, BSO (an inhibitor of GSH synthesis) was used to deplete intracellular GSH. UM-SCC-17B and SCC25CP cell lines were treated with 1 mM BSO for 24 h at 37 °C. Cisplatin (50 μM) was then added for 1 h at 37 °C. Cells were washed twice in PBS and then returned to normal medium. Cells were harvested at 1 and 24 h. Aliquots were used to prepare genomic DNA for platination measurement and to determine GSH levels as described above.

Apoptosis

To determine the relationship between cisplatin exposure, GSH content, and induction of apoptosis, UM-SCC-17B, UM-SCC-38, and SCC-25-CP cell lines were chosen for further study. Double labeling for annexin V and cellular DNA was performed as previously described [15, 26]. Briefly, the cells were seeded at a density of 10^6 per 25 cm^2 in tissue culture flasks, treated with 1 mM BSO for 24 h, and then incubated with cisplatin at a dose approximating the IC_{50} for that cell line. After incubation with the drug for 2 h at 37 °C in culture medium, the cells were washed with PBS and further cultured in drug-free medium for 48 h. Cells (1×10^6) were collected and washed twice in PBS and then stained with propidium iodide and annexin V-FITC (Trevigen, Gaithersburg, Md., USA) in annexin binding buffer at room temperature for 15 min. Samples were diluted with the binding buffer and analyzed by FACscan within 1 h.

Statistical analysis

Linear regression analyses were performed using Prism software (V2.0; Graph Pad Software, San Diego, Calif., USA).

Results

As we have demonstrated previously, glutathione content was positively associated with cisplatin resistance in the panel of cell lines studied (Fig. 1). Cell lines with lower glutathione content were consistently more sensitive to cisplatin than cells with higher glutathione content. In the group of cells studied, a nearly 40-fold range of cisplatin sensitivity was associated with an approximately 4-fold difference in glutathione content.

As expected, modulation of intracellular glutathione content with BSO or NAC resulted in a significant alteration of cisplatin-based cytotoxicity. In Fig. 2, a sensitive (UM-SCC-17B) and resistant (SCC-25CP) tumor cell line were exposed to a dose of cisplatin sufficient to inhibit approximately 50% of cell growth based on prior experiments. For both the sensitive and resistant cell line, reduction in cellular glutathione by treatment with BSO resulted in enhanced cytotoxicity. Conversely, treatment with NAC, which elevates intracellular glutathione, resulted in diminished cell killing at the same dose of cisplatin. Control experiments demonstrated that treatment with BSO reduced detectable glutathione to less than 10% of pretreatment levels. NAC treatment produced more modest increases in glutathione levels of 17–39% above controls (data not shown).

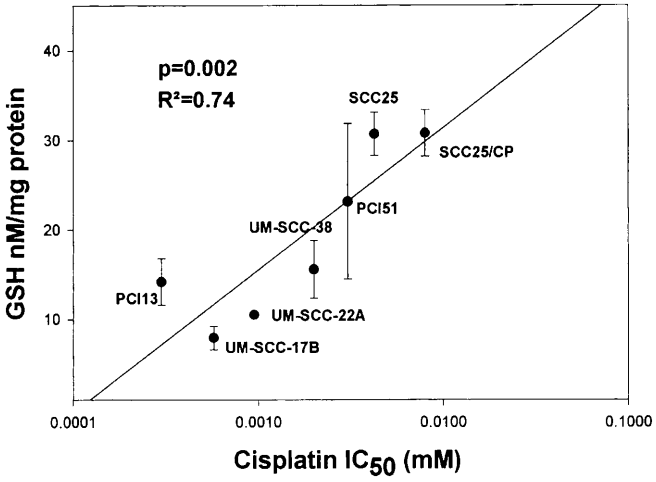

Fig. 1 Cisplatin sensitivity is inversely correlated with intracellular glutathione content in head and neck tumor cell lines. In this panel of seven tumor cell lines, representing a wide range of cisplatin sensitivity, there is a direct correlation between intracellular glutathione content, measured by enzymatic assay, and sensitivity to cisplatin. Cisplatin sensitivity is determined by standard XTT assay

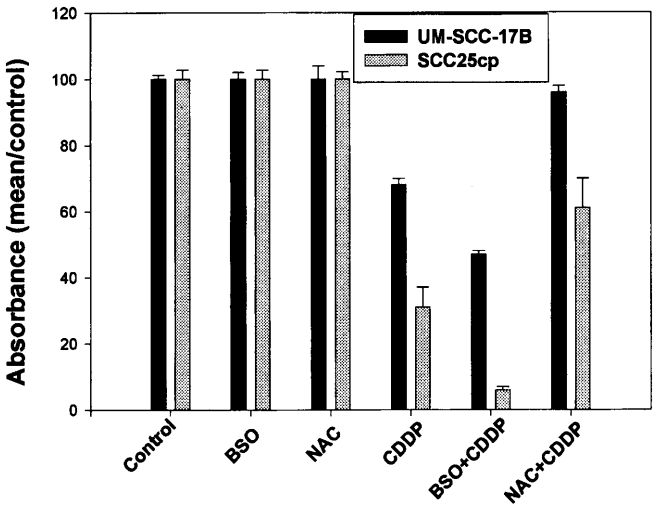

Fig. 2 Modulation of intracellular glutathione content alters cellular sensitivity to cisplatin. In this experiment, sensitive and resistant cell lines were treated with buthionine sulfoximine (*BSO*) or *N*-acetyl-L-cysteine (*NAC*). Treatment with either agent alone had no effect on cell growth measured in this 4-day XTT assay. However, intracellular glutathione was markedly diminished by BSO treatment while treatment with NAC resulted in a less than 40% rise in intracellular glutathione content. As expected, depletion of glutathione enhances cisplatin toxicity in both cell lines while treatment with NAC protects against cisplatin cytotoxicity. *CDDP* Cisplatin

Platinum/DNA adduct levels were then measured by atomic absorption spectroscopy and compared with cisplatin sensitivity in the same panel of cell lines. In this experiment, we were able to demonstrate a significant positive correlation between cisplatin sensitivity and platinum/DNA adduct formation. Cisplatin-sensitive head and neck tumor cell lines had significantly elevated levels of platinum/DNA adducts after 1 h of drug exposure. As expected, the resistant cell lines form significantly fewer adducts after 1 h of drug exposure. The mean of three determinations is shown (Fig. 3).

Surprisingly, when glutathione content was compared directly to platinum/DNA adduct formation, no significant correlation was seen (Fig. 4). Despite the inverse correlation between glutathione content and cisplatin sensitivity as well as the correlation between platinum/DNA adduct formation and cisplatin sensitivity, there was no direct correlation between adduct formation and intracellular glutathione content. This result suggests that glutathione did not directly impact the quantity of platinum/DNA adduct formation in this short-term assay.

The further suggestion that glutathione may have an indirect impact on platinum/DNA interactions can be seen in an analysis of platinum/DNA adduct repair. In this experiment (Fig. 5), the formation and rate of repair of platinum/DNA adducts were measured in a resistant and a sensitive head and neck tumor cell line. The sensitive cell line (UM-SCC-17B) forms approximately twice as many platinum/DNA adducts after 1 h of drug exposure when compared with the resistant tumor cell line (SCC-25CP). Additionally, at the end of 24 h, the resistant tumor cell line is able to repair approximately 70% of measured platinum/DNA adducts while the sensitive cell line reduces the content of measured adducts by less than half. In the experiment shown, the number of UM-SCC-17B cells increased 29% over

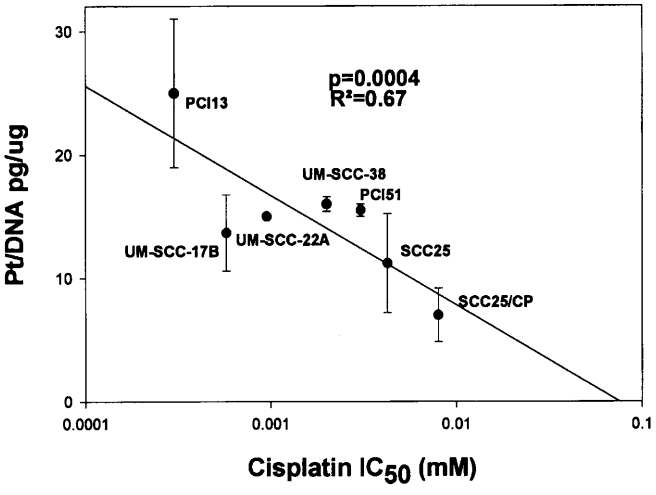

Fig. 3 Cisplatin resistance is inversely correlated with platinum/DNA adduct formation. In this experiment, the seven head and neck tumor cell lines were treated with an equivalent dose (50 μM) of cisplatin. After 1 h, DNA was harvested and platinum/DNA adducts were measured directly by atomic absorption spectroscopy. The most sensitive cell lines showed significantly increased platinum/DNA adduct formation compared with resistant tumor cell lines

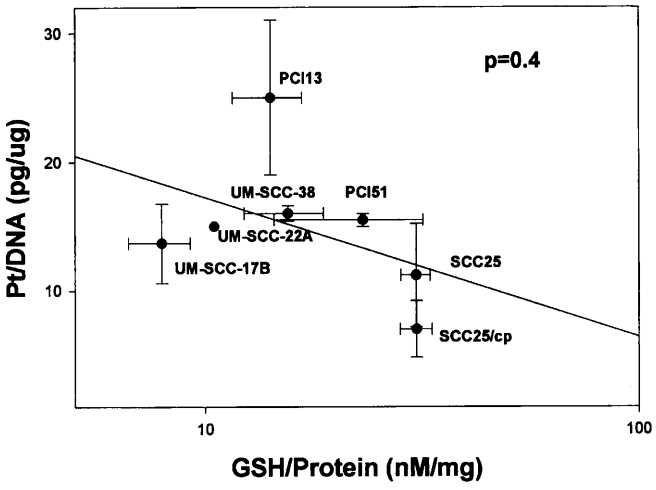

Fig. 4 Platinum/DNA adduct formation does not correlate with intracellular glutathione. The panel of head and neck tumor cell lines was treated with cisplatin as discussed in Fig. 3. Immediately prior to cisplatin treatment, parallel flasks of cells were harvested and evaluated for intracellular glutathione content by enzymatic assay. There is no statistical correlation between the glutathione content measured and the quantity of platinum/DNA adduct formed at an equivalent dose of cisplatin (50 μM). The mean of three experiments is shown

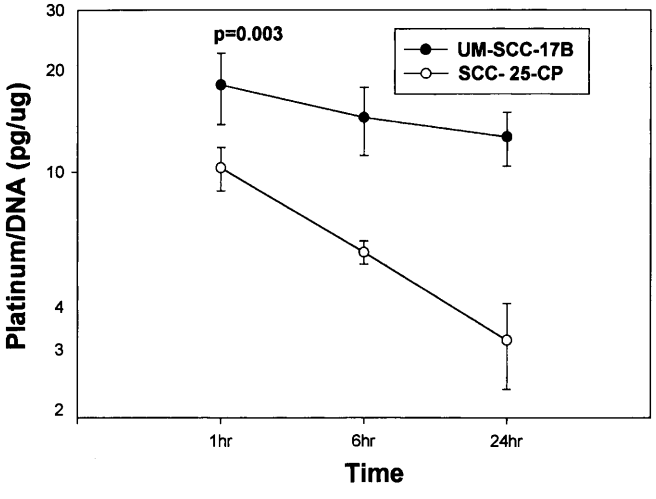

Fig. 5 Platinum/DNA adduct repair is increased in resistant cells. In this experiment, a resistant cell line and a sensitive cell line were exposed to an equivalent dose of cisplatin for 1 h. The cells were then washed and plated on normal medium. Platinum/DNA adducts were measured in flasks at the time points shown. The resistant cell line (SCC-25CP) formed fewer adducts and was able to repair approximately 70% of those adducts over 24 h when compared with the sensitive cell line (UM-SCC-17B) which repaired only 9–28% of adducts in the same time period. In the experiment shown, the number of UM-SCC-17B cells increased 29% over baseline in the first 24 h after platinum exposure, while the SCC-25CP cells increased 20% in the same period. Therefore, the difference in rate of reduction of measured adducts is not accounted for by differences in cell proliferation and DNA synthesis

baseline in the first 24 h after platinum exposure, while the SCC-25CP cells increased 20% in the same period. Therefore, the difference in rate of reduction of measured adducts is not accounted for by differences in cell proliferation and DNA synthesis.

When cellular glutathione content was reduced through treatment with BSO, there was only a modest increase in the quantity of adducts formed after 1 h of cisplatin treatment, despite the fact that glutathione had effectively been eliminated from the cell. In the three cell lines used in this experiment which represent a range of cisplatin sensitivity, complete or near complete depletion of glutathione resulted in only a modest increase in cisplatin adducts (one-third or less). Interestingly, in this experiment, adduct repair measured after 24 h did not change significantly as a result of glutathione depletion (Fig. 6).

The influence of glutathione on sensitivity to apoptosis was measured directly by flow cytometry with cells labeled with propidium iodide and annexin V. In this experiment, depletion of glutathione was accompanied by a significant increase in the proportion of cells undergoing apoptosis at the same dose of cisplatin. BSO treatment resulted in a 1.7- to 2.2-fold increase in the proportion of cells undergoing apoptosis at 48 h (Fig. 7). The increase in cells undergoing apoptosis was most pronounced in the resistant cell line SCC-25CP.

Discussion

The data presented support the hypothesis that cisplatin-induced cytotoxicity in head and neck tumor cell lines is a direct function of platinum/DNA adduct formation. We have shown a strong positive correlation between adduct formation and cisplatin sensitivity in the panel of seven tumor cell lines tested. As expected, our experiments confirm that modulation of cellular glutathione with BSO or NAC can have a significant effect on overall cisplatin-induced cytotoxicity.

As we have demonstrated previously [20], there is an inverse relation between glutathione content and cisplatin sensitivity in these cells. We also demonstrated a direct correlation between cisplatin sensitivity and cisplatin/DNA adduct formation. Somewhat surprisingly, despite the correlation between cisplatin sensitivity and glutathione content, there was no direct correlation between the formation of platinum/DNA adducts and total cellular glutathione measured in short-term assays. We also demonstrate that resistant cells have a greater ability to repair adducts, but that this repair was not dramatically impacted by modulation of glutathione.

However, depletion of glutathione did significantly increase the sensitivity to apoptosis at a given dose of cisplatin (especially in resistant cells) despite a very modest increase in adduct formation and no significant impact on the rate of adduct repair. These results suggests that the protective effect of glutathione on cisplatin cytotoxicity involves a distal cellular response pathways once adducts are formed, and that the impact of glutathione on these downstream pathways is greater than its impact on the more proximal events of adduct formation and repair.

Recent published data support these findings and the notion that glutathione's impact on resistance is mediated by downstream mechanisms. A study of head and neck tumor cell lines produced results similar to those shown in Fig. 3 and the authors concluded that cisplatin-induced growth inhibition does correlate with cellular platinum levels [28]. Other analyses have demonstrated that depletion of intracellular glutathione by BSO has variable impact on cisplatin cytotoxicity [17, 19]. In the latter study of lung cancer cell lines, GSH depletion by BSO treatment did not significantly change measured platinum/DNA binding. Similarly, a recent study in a human melanoma system demonstrated that depletion of glutathione by BSO had no effect on the short-term accumulation of platinum within the cell or on platinum/GSH conjugates [22].

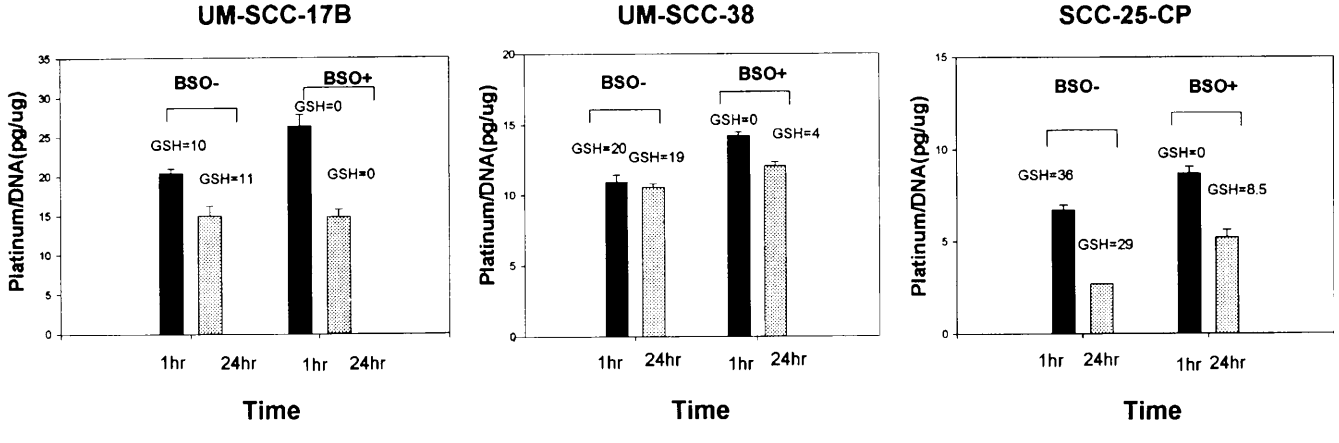

Fig. 6 Glutathione depletion results in a modest increase in platinum/DNA adduct formation. In this experiment, platinum/DNA adducts were measured in three cell lines in the presence or absence of BSO. BSO treatment resulted in near complete depletion of glutathione *S*-transferase (*GSH*) in all cell lines, but this was accompanied by a relatively modest increase in the formation of platinum/DNA adducts (29–42% compared with untreated cells). The rate of repair of platinum/DNA adducts was not significantly changed by treatment with BSO. GSH levels for each sample are shown above the corresponding *bar*

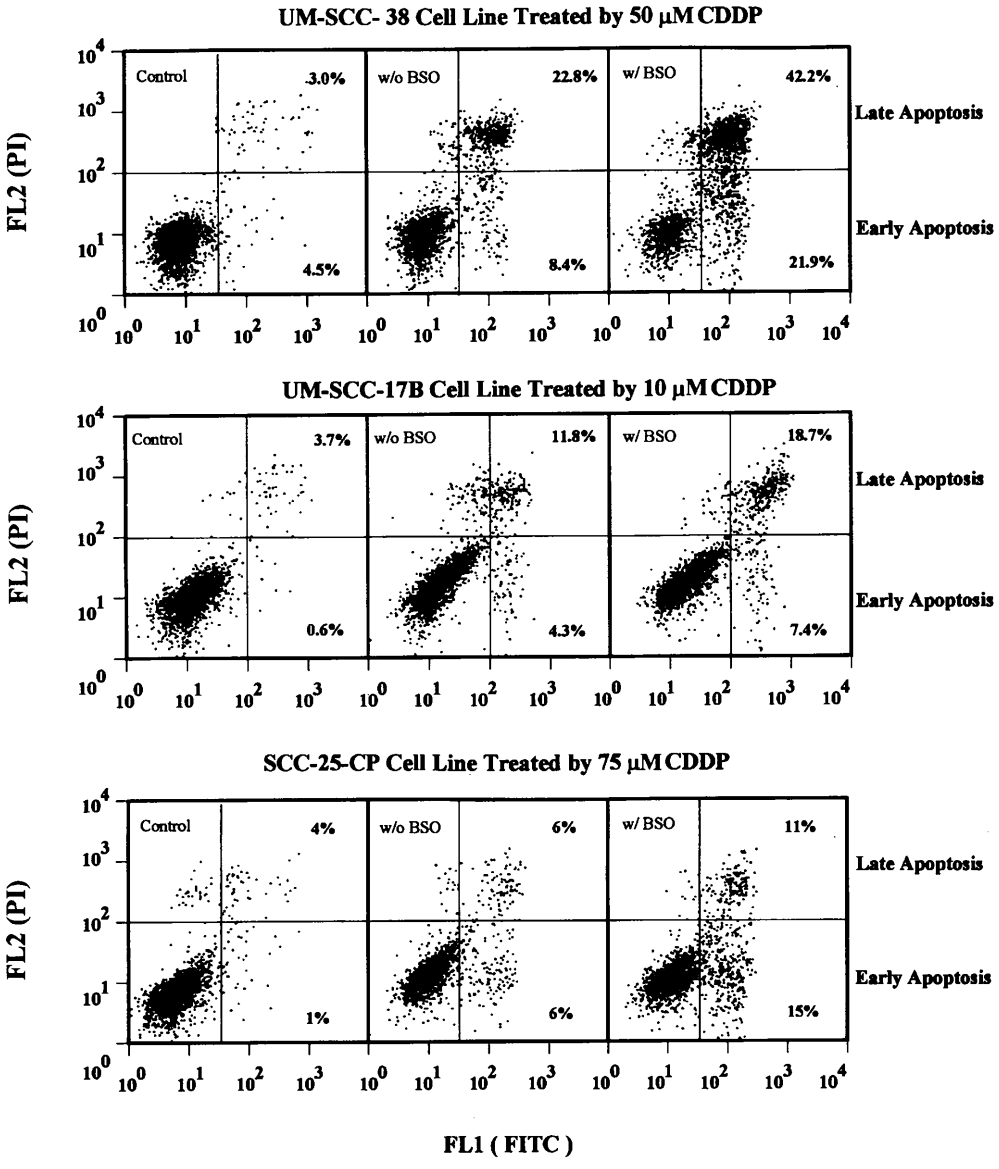

Fig. 7 Glutathione depletion with BSO results in a significant increase in apoptosis. In this experiment, cells were treated with cisplatin at the doses shown in the presence or absence of BSO. The same dose of cisplatin in the presence of BSO resulted in a significant increase in cells undergoing apoptosis as measured in this study utilizing cells labeled with both annexin V and propidium iodide (*PI*). *FL1* Fluorescence channel 1 represents the intensity of staining of annexin V (the antibody which detects apoptosis), *FL2* fluorescence channel 2 represents the intensity of propidium iodide (representing the total number of cell nuclei)

These results, combined with our data, are inconsistent with the long-standing hypothesis that glutathione protects against cisplatin cytotoxicity by formation of glutathione/cisplatin conjugates which are then exported from the cell by means of an ATP-dependent pump mechanism [8]. Collectively, these studies and our data suggest that glutathione may modulate the cytotoxicity of platinum compounds by a means other than simply the formation of glutathione/platinum conjugates which are unable to bind DNA and are subsequently exported from the cell. These glutathione-influenced mechanisms appear to influence sensitivity to apoptosis, since adduct formation and DNA repair are not significantly altered by glutathione depletion.

Recently, several studies have provided insight into potential mechanisms which define interactions between glutathione and apoptotic pathways. Transfection studies have shown that Bcl-2 overexpression, which normally inhibits apoptotic pathways, is accompanied by an increase in total cellular glutathione [18]. Depletion of glutathione caused cells which overexpressed Bcl-2 to become sensitive to apoptosis even though Bcl-2 levels did not change directly. The hypothesis generated by this study is that Bcl-2 may not directly regulate sensitivity to apoptosis but rather acts, at least in part, by way of an intermediary molecule such as glutathione.

More recently, the same group demonstrated that overexpression of Bcl-2 was accompanied by a relocalization of glutathione to the nucleus from the cytoplasm of the cell. Nuclear Bcl-2 protein levels were directly correlated with nuclear glutathione content [27]. The authors hypothesize that the increase in nuclear glutathione content may be important in inhibiting nuclear enzymes (collectively known as caspases) which directly lead to DNA fragmentation in apoptosis. Inhibition of these enzymes by glutathione may be a critical mechanism by which Bcl-2 inhibits apoptosis. Similarly, depletion of glutathione in radiation-resistant cells which

overexpress Bcl-2 results in reversal of radiation resistance [18].

Treatment of tumor cells with antioxidants such as NAC produces increased cellular glutathione levels (decreasing cisplatin sensitivity) but is also associated with increased Bcl-2 protein expression. In a study of colon tumor cell lines, treatment with NAC increased intracellular glutathione, but also increased Bcl-2 protein expression as much as fourfold. Predictably, this was accompanied by acquired resistance to chemotherapy agents [7].

However glutathione is acting, the results of these studies do not exclude the possible importance of other mechanisms, such as metallothionein pathways, which may prevent DNA damage by cisplatin and similar agents.

Despite the fact that cisplatin has been in clinical use for well over 20 years, our understanding of the cellular mechanisms which determine its efficacy or lack thereof in treating an individual tumor are still evolving. Our studies continue to support the hypothesis that glutathione is a critical determinant of cisplatin resistance in these tumors, but perhaps through pathways which determine the cells response to platinum/DNA adducts rather than the formation of those adducts themselves. Further studies will better elucidate the cellular impact of glutathione in clinical drug resistance and clarify optimal targets for therapeutic intervention.

Acknowledgements This research was supported by NCI grant R21CA73866.

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