Selective Modulation of Glutathione Levels in Human Normal versus Tumor Cells and Subsequent Differential Response to Chemotherapy Drugs

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ABSTRACT

Cellular glutathione (GSH) levels were found to be 7-fold higher in a human lung adenocarcinoma cell line (A549) than in a normal human lung fibroblast line (CCL-210). Differential modulation of cellular GSH was explored in these cell lines by (a) stimulation of GSH synthesis by oxothiazolidine-4-carboxylate (OTZ) and (b) inhibition of GSH synthesis by buthionine sulfoximine (BSO). In the tumor cell line, OTZ treatment had no effect; however, GSH levels of 140–170% of control were achieved in the normal fibroblast line. With BSO, the normal cell line was depleted of GSH at a faster relative rate than with the tumor line. Within 7 h, 5% GSH remained in the CCL-210 line while approximately 40% GSH remained in the A549 line.

Survival response of normal versus tumor cell lines to selected chemotherapy was compared following modulation of GSH levels. OTZ pretreatment of the A549 line provided no protection to a 1-h exposure to melphalan, cisplatin, or bleomycin; however, OTZ pretreatment of CCL-210 elevated GSH and provided protection to melphalan, cisplatin, and bleomycin (protection ratios at 5% survival of 1.2, 1.4, and 1.4, respectively). Neocarzinostatin toxicity in the normal CCL-210 line pretreated with BSO was greatly reduced (protection ratio at 50% survival = 5.0). The same BSO treatment to A549 cells (40% GSH remaining) yielded a similar survival curve to control cells.

These studies demonstrate that selective differential chemotherapy responses of normal versus tumor cells is possible by manipulating the GSH synthetic cycle. Should basic phenotypic differences with regard to reductive capacity exist in vivo, such manipulation in GSH levels might yield a therapeutic gain for carefully selected chemotherapy drugs.

INTRODUCTION

A major problem in the use of chemotherapeutic agents in cancer treatment is the vanishingly small therapeutic index which presents a particular clinical problem as normal tissue damage is often, if not always, dose limiting. Over the years, considerable attention has been focused in developing means to potentiate the cytotoxic effects of chemotherapy drugs (1–6). While this approach is understandable and appropriate, if normal tissue damage is also potentiated, no therapeutic gain or advantage will be realized. In an ideal sense, what is needed are strategies that would produce preferential protection of normal tissues or preferential sensitization of tumor cells. Realization of this ideal most certainly would rely on the exploitation of basic phenotypic or genetic differences between normal and tumor tissues.

There has been increasing evidence that cellular GSH alters the cytotoxicity of many chemotherapy drugs (1, 2, 5) or radiation (7). GSH, the major nonprotein cellular thiol is responsible for a variety of cellular functions including protection from toxic oxygen species and detoxification of various xenobiotics (8, 9). The ability to modulate cellular GSH levels to probe the importance of this tripeptide in various drug or radiation interactions has come from the elegant work of Meister and Griffith (10) and Williamson et al. (11) who have introduced compounds that either inhibit or stimulate GSH synthesis.

When GSH synthesis is inhibited by BSO (10), the effectiveness of several chemotherapy drugs has been increased (1, 2); conversely, when GSH levels were elevated by OTZ, a compound that stimulates GSH synthesis (11), protection against bleomycin and Adriamycin has been reported (1, 2).

In a recent study from our laboratory, it was observed that human tumor cell lines generally had higher levels of cellular GSH than did normal human fibroblasts (12). This finding was of particular interest in light of previous studies from our laboratory demonstrating that GSH synthesis in Chinese hamster cells was stimulated by OTZ. OTZ is converted intracellularly by the action of 5-oxo-L-prolinase to cysteine (11). The cysteine is then rapidly utilized in the GSH cycle with subsequent elevation in GSH levels (1, 2, 12–15). When Chinese hamster cells are exposed to OTZ, there is a rapid elevation in GSH; however, by 2 h, GSH levels plateau at approximately 200% of control. The leveling off in GSH levels is probably as a result of GSH feedback inhibition of γ-glutamylcysteine synthetase and thus regulation of the GSH cycle (15). We questioned if the relatively high GSH levels observed in the human tumor lines represented maximum synthesis and whether these tumor cells could not be stimulated to produce higher levels of GSH by OTZ treatment; additionally, the normal fibroblast lines which are low in GSH compared to the tumor lines might possibly be stimulated by OTZ treatment. Should such differential manipulation in GSH levels in tumor versus normal cells be possible, then the possibility of selective protection of normal cells to selected drugs may be feasible; conversely, due to the relatively high levels of GSH in these tumor lines, it also seemed reasonable to question if GSH synthesis were inhibited by BSO, would GSH levels drop faster in the normal versus the tumor cell line?

The present study demonstrates that selective elevation in GSH levels in a human lung fibroblast cell line compared to a human lung adenocarcinoma cell line is achievable by OTZ treatment; additionally, the relative rate of GSH depletion by BSO treatment was observed to be faster in the normal versus the tumor cell line. The subsequent response to selected chemotherapy drugs on the basis of cell survival following differential manipulation of GSH levels in these cell lines will be presented.

MATERIALS AND METHODS

Cell Culture. Both cell lines used in this study were obtained from American Type Culture Collection, Rockville, MD. Normal human lung fibroblast cells (CCL-210) were grown in F12 medium supplemented with 20% (non-heat inactivated) fetal calf serum, penicillin, and streptomycin. All studies using these cells were conducted between passages 4 and 8 where plating efficiencies ranged from 30–50%.

Human lung adenocarcinoma cells (A549, CCL-185) were grown in...
RPMI medium supplemented with 10% fetal calf serum, penicillin, and streptomycin. Plating efficiencies for A549 cells ranged from 45–85%. Both cell lines were grown as stock cultures maintained at 37°C in an atmosphere of 5% CO2-95% air at pH 7.3.

Drug Exposure. BSO was obtained from Chemical Dynamics. OTZ was synthesized according to the procedure of Keneko et al. (16). Chemical characterization of OTZ is described elsewhere (13). Bleomycin was purchased from Sigma, St. Louis, MO. Melphalan (NSC-8806), cis-diaminedichloroplatinum(II) (NSC-119875), and necarzinostatin (NSC-157365) were kindly supplied by the Drug Synthesis and Chemistry Branch of the National Cancer Institute.

Cells were trypsinized from stock cultures, rinsed, and plated into a number of 100-mm plastic Petri dishes (5 × 10^5 cells/dish) for 48 h prior to drug treatment. The medium was changed 12–16 h prior to drug exposure with a known volume of fresh prewarmed medium. Concentrated stock drug solutions made up in medium were prepared immediately prior to each experiment and added directly to the plates. Cells were pretreated at 37°C with either 10 mM BSO or 10 mM OTZ before various drug additions. BSO and OTZ remained in the medium for the duration of all drug exposures. Following various preincubation periods with BSO or OTZ, various concentrations of drugs from concentrated stock solutions were added directly to the dishes. All drug exposures were for 1 h at 37°C. Following drug exposure, the cells were rinsed twice with phosphate buffered saline, trypsinized, counted, diluted, and plated for macroscopic colony formation. Each survival estimate was plated in triplicate, and each experimental condition was repeated 2–3 times. Cells were incubated at 37°C for colony formation for 10–14 (A549) and 12–16 days (CCL-210). Following appropriate incubation, macroscopic colonies (>50 cells/colony) were fixed with methanol/acetic acid (3:1), stained with crystal violet, and counted.

Figures 2–5 show representative self-contained experiments.

Differences in survival response of the two cell lines to various drugs were expressed by determining a sensitivity factor (SF). The SF was calculated as follows:

\[
SF = \frac{D_{50} \text{ A549 cells}}{D_{50} \text{ 210 cells}}
\]

where D50 represents the drug concentration resulting in 50% cell survival. SFs were also calculated at the 5% survival level.

**GSH Assay.** For GSH measurements, cells were treated as described above. After various incubation times with BSO or OTZ, the cell monolayer was rinsed twice with cold phosphate buffered saline and 2.5 ml cold 0.6% sulfosalicylic acid was added. After 5 min at 4°C, sulfosalicylic acid was removed and total GSH content assayed using the glutathione reductase assay (17). Pretreated BSO and OTZ samples were compared to control samples that had been treated as described above. GSH levels shown on Figs. 1–5 are expressed as the percentage of control values. Absolute GSH values for A549 and CCL-210 cell lines were 325.8 ± 11.6 (SD) and 46.5 ± 6.2 nmol/mg protein, respectively.

**RESULTS**

Differential modulation of cellular GSH in normal human lung fibroblasts compared to human lung adenocarcinoma cells is illustrated in Fig. 1. Fig. 1A shows that exposure of both types of cells to 10 mM OTZ results in markedly different effects in regard to GSH synthesis stimulation. The CCL-210 cells increased in GSH content progressively until leveling off 2–4 h after OTZ addition to 130–150% of control values; however, GSH levels in A549 cells were virtually unaffected by OTZ treatment. When GSH synthesis was inhibited by 10 mM BSO treatment, the extent of GSH depletion relative to control as a function of time was different for the two cell types (see Fig. 1B). After 7 h of exposure to BSO, the CCL-210 line had low GSH levels, which were at or near the lower level of detection for GSH by the assay used. In contrast, GSH depletion relative to control in A549 cells was slower, such that by 7 h of exposure to BSO, 35–45% of cellular GSH was present. In cell survival studies described below, a 2-h preincubation with OTZ or a 7-h preincubation with BSO was utilized. These protocols were selected to optimize the differences between the 2 cell lines in regard to GSH metabolism while minimizing preincubation drug exposure times. While OTZ or BSO pretreatment did not alter plating efficiencies or growth rates for either cell line, it was felt that survival studies should be conducted as soon as either maximum or minimum GSH levels were reached in order to minimize possible indirect cellular effects of altered GSH levels (18).

The effects of differential stimulation of GSH synthesis on A549 versus CCL-210 cells to cisplatin, bleomycin, and melphalan are shown in Figs. 2–4. From these survival curves, sensitivity factors were calculated to compare and contrast the 2 cell lines (see Table 1). A549 cells were found to be more resistant to the cytotoxic effects of melphalan and bleomycin (SF = 3.2 and 3.3–4.0, respectively) than were CCL-210 cells. The increased resistance of A549 cells to these drugs did not completely correlate with inherent GSH levels, as the A549 cells contained approximately 7-fold more GSH than the CCL-210 cells; nonetheless, a definite trend toward resistance to melphalan and bleomycin was observed for the tumor line with elevated GSH. Response to cisplatin and NCS (shown in Fig. 5 and Table 1) was similar in both cell types. When both cell types were pretreated for 2 h with OTZ (Figs. 2–4 and Table 1), modest protection was observed in the CCL-210 cell line wherein GSH had been elevated to 135–171% of control values prior to drug exposure. In contrast, essentially no protection was observed in the A549 tumor line where OTZ pretreatment failed to elevate GSH levels. The OTZ pretreatment preferentially yet modestly protected normal CCL-210 cells exposed to melphalan, bleomycin, and cisplatin.

As shown in Fig. 1B, when each cell line was treated with BSO, the normal fibroblast CCL-210 could be depleted of GSH faster relative to control than the tumor A549 cell line. The survival of A549 and CCL-210 cells following a 1-h exposure to NCS is shown in Fig. 5. If A549 cells were pretreated for 7 h with BSO (GSH = 36% of control) and then treated with
Differential Modulations of Cellular GSH

Table 1 Drug sensitivity factors

<table>
<thead>
<tr>
<th>Drug</th>
<th>Sensitivity factor (control cells)</th>
<th>Sensitivity factor (OTZ pretreatment)</th>
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<tbody>
<tr>
<td>Melphalan</td>
<td>3.2 50% 3.3 5%</td>
<td>2.5 50% 2.6 5%</td>
</tr>
<tr>
<td>Cisplatin</td>
<td>0.9 1.3 1%</td>
<td>0.8 50% 1.0 5%</td>
</tr>
<tr>
<td>Bleomycin</td>
<td>3.3 4.0 2%</td>
<td>3.0 50% 2.9 5%</td>
</tr>
<tr>
<td>NCS</td>
<td>1.4 0.9</td>
<td></td>
</tr>
</tbody>
</table>

Fig. 2. Survival of A549 and CCL-210 cells following a 1-h exposure to cisplatin. •, cells pretreated with 10 mM OTZ for 2 h which elevated GSH to 145% of control in the CCL-210 lines but did not significantly elevate the A549 cell line. Bars, SD when larger than symbol.

Fig. 3. Survival of A549 and CCL-210 cells following a 1-h exposure to bleomycin. •, cells pretreated with 10 mM OTZ for 2 h which elevated GSH to 135% of control for the CCL-210 line but did not significantly elevate the A549 cell line. Bars, SD when larger than symbol.

Fig. 4. Survival of A549 and CCL-210 cells following a 1-h exposure to melphalan. •, cells pretreated with 10 mM OTZ for 2 h which elevated GSH to 171% of control for the CCL-210 line but did not elevate the A549 line. Bars, SD when larger than symbol.

NCS, there was no difference in survival (Fig. 5). In marked contrast, a 7-h BSO pretreatment (GSH = 5% of control) of CCL-210 cells followed by NCS exposure resulted in essentially very little NCS toxicity. These data are consistent with our earlier data on Chinese hamster cells that identified GSH as the major thiol required for reduction of NCS to a toxic species (19).

Discussion

These studies demonstrate that cellular GSH can be differentially manipulated in a normal versus tumor cell line. Depending upon the means of GSH manipulation (OTZ or BSO) and the chemotherapy drug selected, protection could be demonstrated in the normal but not in the tumor cell line. Such differential responses would be highly desirable in a clinical setting.

That GSH levels in the normal cell line can be modulated to a greater relative extent by OTZ and BSO than the tumor line studied warrants discussion. There are several factors which govern whether or not OTZ will stimulate GSH synthesis and result in an elevation in GSH levels: (a) OTZ must get into the cell. Based on the charge characteristics of the molecule and the fact that GSH elevation has been observed in a variety of cell types (11, 13, 14), it would seem reasonable that OTZ does in fact enter the cell; however, we cannot exclude the possibility that OTZ did not get into the tumor cell line in the present study; (b) in order for GSH synthesis to be stimulated by OTZ, sufficient 5-oxo-L-prolinase activity is required. There is the possibility that the particular tumor cell line presently studied has compromised oxoprolinase activity. This issue is currently under study in our laboratory; (c) the high GSH levels in the tumor cell line studied probably results in feedback inhibition of the GSH cycle (15). If this is the case, further increase in cysteine as delivered by OTZ could not result in GSH elevation; (d) since cellular protein levels are higher in the tumor cells, there may be a greater demand for cysteine in protein synthesis and thus OTZ utilization is channeled in that direction; and (e) the lack of GSH elevation by OTZ in these tumor cells may reflect various permutations of all the above factors. The normal cell line, however, with much lower cellular GSH levels may be capable of accommodating higher GSH levels before feedback inhibition occurs.

These various factors concerning the differential modulation in GSH levels of the normal cell line using OTZ and BSO can be systematically studied experimentally. Perhaps an equally important consideration is whether normal versus tumor tissues in vivo can be manipulated in a similar manner. OTZ treatment of mice has been shown to elevate GSH in normal liver and was predicted to elevate GSH in other normal tissues (11). Several laboratories have demonstrated that BSO will deplete murine tumors of GSH to 20–30% of control levels (20). Future in vivo studies in this laboratory will be directed toward a thorough study of elevation (OTZ) and depletion (BSO) rates of tumor and a variety of normal tissues.
Conceptually, should these *in vitro* observations extend to *in vivo* correlates, chemotherapy drugs or analogues of existing drugs might be synthesized to take advantage of differences in GSH levels or differential manipulation in GSH levels in normal versus tumor tissue. A case in point is the drug NCS, which apparently requires cellular GSH for reduction to the toxic species (19). The development of chemotherapy drugs that are activated by tumor specific properties is indeed an appealing possibility and worthy of continued experimental pursuit.

REFERENCES

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