Intracellular Glutathione Levels in Human Colon Cancer Cells Naturally Resistant to Cross-Linking Agents

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Correlation between sensitivity to two cross-linking agents, 1-(4-amino-2-methylpyridine-5-yl)-methyl-1-3-(2-chloroethyl)-3-nitrosourea (ACNU) and cisplatin (DDP), and intracellular glutathione (GSH) level was investigated for two naturally drug-resistant human colon cancer cell lines in comparison with two drug-sensitive human leukemia cell lines. As a result, a high correlation was observed between them. We also studied the possibility that DL-buthionine-S,R-sulfoximine (BSO), an inhibitor of GSH biosynthesis, can sensitize the cancer cells to these anticancer agents via depletion of intracellular GSH. It was found that BSO potentiated ACNU cytotoxicity against human leukemia K562 cells and DDP cytotoxicity against K562 and human colon cancer WiDr cells. It indicates that cancer cells with higher GSH level are more effectively sensitized by BSO regardless of degree of their intrinsic sensitivity to these anticancer agents. These results suggest that intracellular GSH level is not a common mechanism for natural resistance to cross-linking agents in human colon cancer cells but one of the determinants of sensitivity to these anticancer agents of GSH-rich cells.

**Keywords** — intracellular glutathione level; nitrosourea; cisplatin; colon cancer cell; natural resistance

Introduction

The chemotherapy of solid cancers, particularly colon, pancreatic, renal carcinoma, non-small cell lung carcinoma etc. remains a major clinical problem. Natural resistance of these cancer cells to various chemotherapeutic agents has been pointed out. In this connection, we and other investigators compared cellular basis of drug sensitivity among human colon, pancreatic, renal and leukemia cell lines. In these studies, cell lines derived from solid cancers were found in many cases more resistant to dexamethasone, adriamycin, mitoxantrone, etoposide, cisplatin (DDP), methotrexate, and 1-(4-amino-2-methylpyridine-5-yl)-methyl-3-(2-chloroethyl)-3-nitrosourea (ACNU). It is, therefore, very important to know whether the mechanism for drug resistance of solid cancer cell lines is common to such a wide variety of agents or whether multiple mechanisms of resistance to these agents exist.

Glutathione (GSH) is a predominant component of intracellular non-protein sulphydryl compounds that play a part in detoxification and in the repair of cellular injury caused by diverse agents such as nitrogen mustard, melphalan, cyclophosphamide, nitrosoureas, quinine antibiotics (including adriamycin, daunorubicin and mitomycin C). Recently, involvement of GSH in DDP-resistance was reviewed and an increase in cellular GSH in DDP-resistant cells was shown by several investigators. Elevation of the intracellular GSH level was also documented in nitrogen mustard- and melphalan-resistant cells and depletion of cellular GSH by nutritional deprivation of cysteine or by a specific inhibitor of GSH synthesis, DL-buthionine-S,R-sulfoximine (BSO), could potentiate the cytotoxicity of the alkylating agents. Hamilton et al. also reported augmentation of adriamycin, melphalan, and DDP cytotoxicity in drug-resistant and -sensitive human ovarian carcinoma cell lines by BSO mediated GSH deple-

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tion.

As for nitrosoureas, GSH is thought to play a role in detoxification of this class of agents through GSH transferase mediated denitrosation reaction. However, slight potentiation of 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) cytotoxicity by depletion of cellular GSH level was reported. In this report, correlation of cellular sensitivity to ACNU and DDP with the intracellular GSH level was studied on two human colon cancer cell lines and two human leukemia cell lines. We also investigated the possibility that BSO can sensitize the colon cancer and leukemia cells to these two agents.

Materials and Methods

Chemicals — ACNU and DDP were generously supplied by Sankyo Co., Ltd., Tokyo and Bristol-Myers Research Institute, Tokyo, respectively. BSO was purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). All other chemicals were of the highest grade available.

Cells and Culture Conditions — Human chronic myelogenous leukemia K562, acute lymphoblastic leukemia (B-cell type) CCRF-SB, human colon carcinoma DLD-1 and WiDr cell lines were involved in this study. CCRF-SB and K562 cells were grown in a suspension in Roswell Park Memorial Institute Medium 1640 (RPMI 1640) supplemented with 10% fetal calf serum. DLD-1 and WiDr cells were grown in monolayer culture in RPMI 1640 medium for DLD-1 and a minimum essential medium for WiDr supplemented with 10% fetal calf serum. All the cells were cultured in medium containing 100 μg/ml of kanamycin at 37 °C in the humidified atmosphere of 5% CO₂ and 95% air.

Drug Sensitivity — Drug sensitivity was determined by inhibition of cell growth after 1 h exposure of exponentially growing cells to drugs. Leukemia cells were incubated with drugs for 1 h at a density of 5 × 10⁴ cells/ml, washed twice with Hanks’ salt solution and cultured in drug-free medium for 48 h. Then the cell number was determined in a Coulter counter model ZBI (Coulter Electro).

Colon carcinoma cells were plated in 35-mm dishes and incubated for 1 d in order to introduce them into an exponentially growing phase. On the following day of seeding, the cells were treated with drugs for 1 h and washed twice with saline and cultured in drug-free medium for 48 h. The cells were then trypsinized and the cell number was estimated in the Coulter counter. The assays were performed in triplicate and the number of cells in the treated culture was expressed as % of control and the IC₅₀ (the 50% inhibitory concentration) was estimated from a dose–response curve.

GSH Depletion by BSO — Potentiation of cytotoxicity of drugs and GSH estimations were performed on cells which had been preincubated for 23 h in a medium containing various concentrations of BSO. For sensitization assays, the drug solution was added to the culture medium after the cells had been preincubated with BSO for 23 h, and the cells were then incubated with the drug and BSO for 1 h. The cells were washed twice and further cultured in BSO containing and drug-free medium for 48 h. The cell number was determined by the Coulter counter.

GSH Assay — Cultured cells (10⁶—10⁷ cells) were harvested by treatment with trypsin for CCRF-SB and K562 or trypsin/ethylenediaminetetraacetic acid (EDTA) for DLD-1 and WiDr, and were washed with phosphate-buffered saline (PBS). The cells were centrifuged at 2000 g for 5—7 min. The precipitates were resuspended in 2 ml of distilled water and were homogenized on ice using a glass homogenizer. The homogenates were centrifuged at 10000 g for 5 min and the resulting supernatants were centrifuged at 105000 g for 60 min. The obtained cytosolic fraction was centrifuged with 8% sulfosalicylic acid at 10000 g for 10 min to remove cellular protein.

GSH was measured enzymatically according to the method of Tietze as modified by Griffith, consisting of a cyclic reduction of GSH with nicotinamide adenine dinucleotide phosphate (NADPH) and GSH reductase, and concurrent color change of the reagent 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB). Three working solutions were made up in stock buffer (125 mM Na-phosphate, 6.3 mM Na-EDTA to pH 7.3) as follows; (1) 0.3 mM
NADPH, (II) 6 mM DTNB and (III) approximately 50 units of GSH reductase/1 ml.

To assay for total GSH, 1.4 ml of solution I, 200 μl of solution II, and the cytosolic fraction or control to give a final volume of 2.0 ml were mixed in a test tube and equilibrated to 30 °C. This solution was added to 20 μl of the warmed solution III, and after mixing transferred from the test tube to the cuvette, and the A_{412} was monitored continuously until it exceed 2.0. The GSH content of the aliquot assayed is determined by comparison of the rate observed to a standard curve.

**Results**

**Correlation of Cellular Sensitivity to ACNU and DDP with Intracellular GSH Contents**

Sensitivities to ACNU and DDP were compared by IC_{50} values between human colon cancer and leukemia cells. Colon cancer cells exhibited 22—27 fold resistance to ACNU and 4—7 fold resistance to DDP as compared with leukemia cells (Table I).

To investigate mechanism for this natural resistance to ACNU and DDP in the colon cancer cells, intracellular GSH levels of DLD-1 and WiDr were compared with those of CCRF-SB and K562. As shown in Table I, DLD-1 cells were the most resistant to ACNU and DDP of the four cell lines studied. However, intracellular GSH level of DLD-1 cells was the lowest. Furthermore, K562 cells, which were next to the most sensitive to ACNU and DDP showed the highest level of intracellular GSH. Therefore, no correlation between drug sensitivity and intracellular GSH level was observed in the cell lines studied.

These results suggest that intracellular GSH level is not a common mechanism for natural resistance to cross-linking agent of colon cancer cells.

**Correlation between Intracellular GSH Level and Sensitivity to BSO**

Cellular sensitivity to BSO was investigated in relation to intracellular GSH level. As shown in Table II, a relatively high sensitivity to growth inhibitory effect of BSO was observed in DLD-1 and CCRF-SB cells, with the relatively low levels of intracellular GSH of the four cell lines studied. Table II also indicates that intracellular GSH levels of DLD-1 and CCRF-SB cells were significantly suppressed by as low a concentration as 5—10 μM BSO, while 50 and 500 μM

### Table I. Sensitivities to ACNU and DDP of Human Cancer Cell Lines and Their Intracellular GSH Levels

<table>
<thead>
<tr>
<th>Kind of cancer</th>
<th>Cell line</th>
<th>IC_{50} (μM) ACNU</th>
<th>IC_{50} (μM) DDP</th>
<th>Intracellular GSH level (nmol/10^{6} cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leukemia</td>
<td>CCRF-SB</td>
<td>4.8 (1.0)^a</td>
<td>4.0 (1.0)</td>
<td>3.4</td>
</tr>
<tr>
<td></td>
<td>K562</td>
<td>6.6 (1.4)</td>
<td>4.8 (1.2)</td>
<td>10.7±4.7</td>
</tr>
<tr>
<td>Colon</td>
<td>WiDr</td>
<td>105 (22)</td>
<td>14 (3.5)</td>
<td>9.2±2.1</td>
</tr>
<tr>
<td></td>
<td>DLD-1</td>
<td>128 (27)</td>
<td>29 (7.3)</td>
<td>1.8±0.096</td>
</tr>
</tbody>
</table>

*a) Numbers in parentheses are ratio of IC_{50} values to that of CCRF-SB cells.

### Table II. Sensitivity to BSO of Human Cancer Cell Lines

<table>
<thead>
<tr>
<th>Cell line</th>
<th>IC_{50} (μM) for cell growth^a</th>
<th>Suppression of intracellular GSH level</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>BSO concentration (μM)</td>
</tr>
<tr>
<td>DLD-1</td>
<td>13</td>
<td>10</td>
</tr>
<tr>
<td>CCRF-SB</td>
<td>68</td>
<td>5</td>
</tr>
<tr>
<td>WiDr</td>
<td>1600</td>
<td>500</td>
</tr>
<tr>
<td>K562</td>
<td>1100</td>
<td>50</td>
</tr>
</tbody>
</table>

*a) Cells were cultured with various concentrations of BSO for 72 h and cell number was counted by a Coulter counter.
Glutathione and Drug Resistance

Fig. 1. Effects of BSO on Sensitivities to ACNU and DDP of K562 and WiDr Cells

K562 cells (A and B) and WiDr cells (C and D) were preincubated with 50 and 500 μM BSO, respectively, (●) or without (○) it for 23 h and treated with ACNU (A and C) or DDP (B and D) for 1 h. Cells were then further cultured with or without BSO for 48 h. Each value shows percentage of cell number of control. Points are the mean values of triplicates with coefficient of variation less than 14%.

BSO were needed to induce the similar degree of suppression in K562 and WiDr cells, respectively. These results indicate that growth of GSH-poor cells were very sensitive to reduction of intracellular GSH level by BSO.

Effects of BSO on Sensitivities to ACNU and DDP of K562 and WiDr Cells

To determine how much the intracellular GSH level is involved in sensitivities to ACNU and DDP in GSH-rich cells (K562 and WiDr cells), effects of BSO on sensitivities to ACNU and DDP of these cell lines were studied. For K562, significant sensitization of ACNU- and DDP-sensitivities were observed. On the basis of IC₅₀, 9- and 6-fold increases in sensitivity to ACNU and DDP were accomplished (Fig. 1A and B). This result suggests that GSH plays a significant role in sensitivities to ACNU and DDP of K562. On the other hand, DDP sensitivity of WiDr cells was significantly augmented (14-fold) by BSO but the inhibitor of GSH-biosynthesis had no effect on ACNU sensitivity of WiDr cells (Fig. 1C and D). Therefore, for WiDr cells, intracellular GSH level is one of determinants of DDP-sensitivity. In contrast, BSO had little effect on GSH-poor cells (DLD-1 and CCRF-SB) to both ACNU and DDP (data not shown).

Discussion

The purpose of the present study is to investigate the possibility that intracellular GSH level contributes to natural resistance to cross-linking agents in human colon cancer cells as one of the common mechanisms. However, the intracellular GSH level was not necessarily higher in resistant colon cancer cell lines in comparison with sensitive leukemia cells. Indeed, DLD-1 cells, which were the most resistant to ACNU and DDP of the cell lines studied exhibited the lowest level of GSH, suggesting other mechanisms of resistance than GSH.

As for ACNU resistance, an elevated rate of removal of alkyl moiety from O⁶-alkylguanine was demonstrated in ACNU-resistant Chinese hamster cell lines¹⁵) and human glioma cell lines.¹⁶) The chloroethynitrosourea-resistant human glioma cells were reported to be equally sensitive to DDP and more sensitive to nitrogen
mustard compared with ACNU-sensitive glioma cells, indicating that the molecular mechanisms of cellular resistance to chloroethyl-nitrosoureas do not result in cross-resistance to nitrogen mustard or DDP.\(^6\)

With regard to acquired resistance to DDP, reduced uptake of DDP,\(^7\) increase in unscheduled deoxyribonucleic acid (DNA) synthesis after treatment with DDP,\(^6\) elevation of GSH,\(^5\) and GSH-related enzymes\(^5\) have been reported. In view of such diverse mechanisms for the acquisition of resistance to nitrosoureas and DDP, it is remarkable that human colon cancer cells are naturally resistant to a wide variety of chemotherapeutic agents including ACNU and DDP.

Therefore, reversal of drug resistance is very important, so that potentiation of melphalan and nitrogen mustard cytotoxicity by BSO has been reported by a number of investigators.\(^8\)–\(^12\)

On the other hand, only slight augmentation of cellular sensitivity to BCNU\(^11\) and DDP\(^7,10\) by BSO was reported. On the contrary, Hamilton\( et\, al.\)\(^12\) showed augmentation of DDP by BSO.

We also showed potentiation of DDP cytotoxicity against K562 and WiDr cells and of ACNU cytotoxicity against K562 cells by BSO. Our results suggest that GSH-rich cells have greater tendency to be sensitized by BSO than GSH-poor cells regardless of their sensitivities to these cross-linking agents and thus intracellular GSH level is one of determinants of sensitivity to this class of antitumor agent in these cells. Concerning the treatment schedule of BSO, Richon\( et\, al.\)\(^7\) and Andrews\( et\, al.\)\(^10\) treated tumor cells with BSO only before treatment of the cells with DDP. On the contrary, we and Hamilton\( et\, al.\)\(^12\) treated cells with BSO not only before DDP treatment but also after addition of DDP. Considering these presentations, GSH may play an important role in process after the DNA has been platinated as well as in an inactivation process through production of drug–GSH conjugates. However, in the post-platination process it remains to be determined whether they involve DNA repair or some other unknown mechanism. This can be also the case in ACNU sensitivity in K562.

There are a number of problems to be solved in elucidating mechanisms of drug resistance in human colon cancer cells. Our efforts are now directed toward studies on inhibition of DNA repair in human colon cancer cells.

References


