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Effects of Buthionine Sulfoximine Treatment on Cellular Glutathione Levels and Cytotoxicities of Cisplatin, Carboplatin and Radiation in Human Stomach and Ovarian Cancer Cell Lines*

— Glutathione, Buthionine Sulfoximine, Cytotoxicity —

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Chemotherapy failure remains a significant medical problem in the treatment of neoplastic disease and is thought to be due to many different factors including membrane transport, p-glycoprotein in multidrug resistance, glutathione and its related enzymes, topoisomerase II and DNA repair.

Glutatione is a major constituent of non-protein thiol and participates in detoxification of chemotherapy and radiation. Thus, glutathione concentration is correlated with sensitivity to alkylating agents and radiation, and increased in resistant cell lines. Buthionine sulfoximine (BSO) is an inhibitor of glutathione biosysthesis and may increase cytotoxicities of alkylating agents, including melphalan and cisplatin, and radiation in sensitive and resistant cell lines.

We studied effects on cellular glutathione levels and cytotoxicites of cisplatin, carboplatin and radiation by BSO treatment in human stomach cancer cell line (SNU-1) and ovarian cancer cell line (OVCAR-3).

The results were as follow:

1) After BSO treatment of 1 mM and 2 mM for 2 days, the intracellular thiol concentration was depleted to 75.7% and 76.2% in SNU-1, and 74.1% and 63.0% in OVCAR-3, respectively.

2) The intracellular thiol concentration in SNU-1 was depleted to 33.4% after BSO 2 mM for only 2 hours incubation and 71.5% after small amount of BSO (0.02 mM) for 2 days.

3) The recovery of intracellular thiol concentration required more than 3 days after BSO removal.

4) BSO inhibited partially the growth of SNU-1 and OVCAR-3.

5) The cytotoxicities of cisplatin and carboplatin were markedly enhanced both in SNU-1 and OVCAR-3 by BSO treatment.

6) The cytotoxicities of radiation was inceased in OVCAR-3 and SNU-1 by BSO treatment.

Therefore, it is concluded that BSO can deplete effectively the intracellular thiol concentration and enhance the cytotoxicities of cisplatin, carboplatin and radiation.

Key Words: *Glutathione, Buthionine sulfoximine, Cytotoxicity*

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INTRODUCTION

In the past two decades, remarkable accomplishments have been made in the treatment of several types of cancers resulting in many patients being cured. Modern chemotherapeutic regimens are capable of producing long-term remissions and possible cures in patients with Hodgkin's disease, malignant lymphoma, acute leukemias and several solid tumor including testicular cancer and early stage breast cancer. In addition, other malignancies, such as ovarian carcinoma, small-cell lung cancer and advanced breast cancer, while not yet curable, are effectively treated with the use of combination chemotherapy. Unfortunately, when a relapse occur following initial chemotherapeutic responses, it is usually associated with the development of drug resistance (acquired resistance) and responses to additional chemotherapy are less frequent and less durable. And some tumors of the visceral organs (e.g. colon, stomach, pancreas) frequently are not responsive initially (inherent resistance) to chemotherapeutic agents.

The identification of mechanisms of durg resistance is an important goal of current research. Emphasis has been placed on elucidation of specific mechanisms of resistance because this is viewed as the first step toward overcoming drug resistance. One of the systems that has been studied extensively in vitro is multidrug resistance (MDR) mediated by p-glycoprotien. Clinical data indicates that chemosensitizers including verapamil could overcome MDR[1].

The mechanisms responsible for the development of resistance to alkylating agents, only partially characterized, are multifactorial and may involve drug transport, metabolism and/or repair of damaged DNA.

A relationship exists between intracellular glutathione (GSH) levels and cytotoxicities to melphalan, cisplatin and irradiation in human ovarian cancer cell lines. Cell lines with resistance induced in vitro to either melphalan or cisplatin have remarkable elevation in intracellular GSH levels compared to the sensitive cell lines[2]. Furthermore, when GSH levels are lowered with buthionine sulfoximine (BSO), a synthetic amino-acid analog which specifically inhibits gamma-glutamylcysteine synthetase, there is increased cytotoxicity of melphlan and cisplatin in both the drug-sensitive and resistance cell lines. In addition, depletion of GSH is associated with the reversal of cross-resistance to irradiation and some chemotherapeutic agents in cell lines with acquired resistance to either melphalan or cisplatin. If BSO can be safely administered to cancer patients, it may lead to improved therapy in tumors in which there is a steep dose response relationship[3,4].

However it must be emphasized that GSH depletion of normal cells may increase the toxicity of some antineoplastic drugs and irradiation, as well as of those non-cancer drugs in which GSH is required for metabolism and detoxification. The direct toxic effect of BSO is still not well evaluated[5]. Consequently, careful preclinical studies defining optimal concentration and duration of treatment of BSO will be required prior to any clinical trials.

To evaluate the effect of BSO on intracellular GSH concentration and on cytotoxicity, we studied change of intracellular GSH according to the BSO concentration and treatment duration, and effects on cytotoxicitly induced by cisplatin, carboplatin and irradiation after BSO treatment.

MATERIALS AND METHODS

1. Materials

1) Cell lines

SNU-1 is the cell line derived from the patient with stomach cancer[6] and OVCAR-3 is the cell line derived from the malignant ascites of a patient of ovarian adenocarcinoma. Two cell lines are maintained in RPMI 1640 medium containing fetal bovine serum (10%, v/v), penicillin (100,000 unit/10 ml), streptomycin (100 mg/10 ml) and 200 mM L-glutamine. Cultures are maintained at 37°C in humidified atmosphere of 5% CO_2 in air. Passage of the cells are accomplished twice in a week and with trypsin in OVACR-3.

2) Drugs

Cisplatin and carboplatin were obtained from NCI, USA. Cisplatin (10^{-2}M, 2×10^{-3}M) and carboplatin (2×10^{-2}M, 8×10^{-3}M) were dissolved in dimethyl sulfoxide, stored at -20°C, and diluted with medium as necessary. GSH, L-buthionine sulfoximine (BSO) and 3-{4, 5-dimetylthiazole-2-yl}-2, 5-diphenyltetrazolium bromide (MTT) were purchased from Sigma Chemical Co. GSH was dissolved in 6.5% trichloroacetic acid (TCA) at 1

mM. BSO was dissolved in sterile water immediately before study. MTT was dissolved in Hanks balanced salt solution at 5 mg/ml, stored at 4°C and filtered before use.

3) Irradiation

One dose of 30-40 Gy was given at room temperature using 6 MV linear accelerator.

2. Methods

1) Determination of Intracellular Thiol

Cultured cells (10^6 cells/ml) were harvested with pipetting (SNU-1) or trypsin/EDTA (OVCAR-3) and were lysed by 6.5% TCA. Total thiol concentration of supernatant was determined according to Saville[7] [To 0.5 ml of supernatant, add 0.5 ml solution A (sidium nitrite 0.01 M 1 volume, 0.2-1.0 N sulphuric acid 9 volume) and let stand for 5 min. Then add 0.1 ml solution B (0.5% ammonium sulphamate/sterile water) and let stand for 5 min. And add 1 ml solution C (1% mercuric chloride 1 volume, 3.4% sulphanilamide/0.4 N hydrochloric acid 4 volume) and let stand for 5 min. And finally add 0.4 ml solution D (0.1% N-1-naphthyl-ethylenediamine dihydrochloride/0.4 N hydrochloric acid) and let stand for 5 min, then colour will develop.]. The coloured solution was measured against an appropriate blank solution with spectrophotometer at 535 nm. Blank solution was prepred with 6.5% TCA 0.5 ml according to above method. Standard GSH solution was 1 mM GSH in 6.5% TCA. Calibration graphs relating spectrophotometer reading to 5 ul, 10 ul, 20 ul, 50 ul of GSH concentration were plotted (Fig. 1).

2) Determination of Intracellular thiol after BSO Treatment

Intracelluar thiol concentration was determined after BSO treatment according to BSO treatment durations (2 hour, 1 day, 2 days, 3 days) and BSO concentrations (0.02~2 mM) were determined. Recovery of intracellular thiol concentration after BSO removal was observed.

3) Cytotoxicity Assay using MTT Method

Cancer cell lines were treated with BSO (1-2 mM) for 2 days at 37°C incubator. After washing 2 times, cancer cells were treated with irradiation or with cisplatin or carboplatin for 1 hour at 37°C and washed. Single suspensions of OVCAR-3 were plated in 96-well plate (3.5×10^3 cells/135 ul/well) before BSO treatment. SUN-1 were plated in 96-well (2×10^4 cells/135 ul/well) after treatment with BSO and antineoplastic agents. Cancer cells were incubated for 3 days at 37°C, 5% CO_2 incubator. Stock MTT solution 15 ul was added to all wells and incubated for 4 hours in 37°C incubator. After incubation, MTT was removed by suction (SNU-1 was centrifuged for 5 min at 500 rpm before suction.) and DMSO 100 ul was added to dissolve tetrazolium dye on minishaker for 10 min. Within 1 hour the optical density of each well was measured with a microplate spectrophotometer (MR 700 Microplate Reader, Dynatech Lab Inc. USA) at 570 nm. Cytotoxicity was calculated as the ratio of optical denstiy of experiment and control and expressed as % survival[8,9].

$$\% \text{ survival} = \frac{\text{optical density of experiment}}{\text{optical density of control}} \times 100$$

RESULTS

1. Standard calibration gralph of GSH concentration (Fig. 1) and intracellular thiol concentration of cancer cell lines.

2. Intracellular thiol concentrations after BSO treatment.

1) BSO 1 mM and 2 mM treatment for 2 days depleted intracellular thiol concentration 75.7% and 76.2% in SNU-1, and 74.1% and 63.0% in OVCAR-3, respectively (Fig. 2).

2) BSO 2 mM treatment depleted intracellular thiol concentration 33.4% and 76.3% at 2 hours and 1 day, respectively. But there was no more

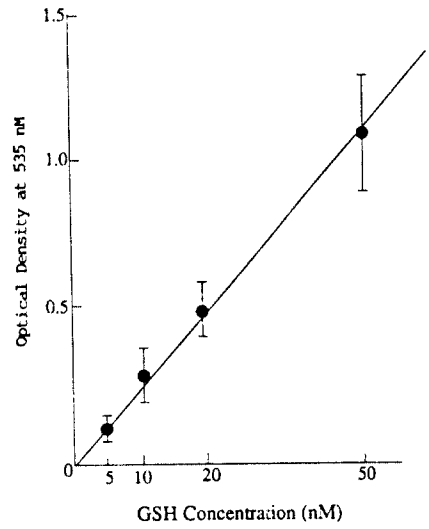

Fig. 1. Cellular GSH Calibration Graph obtained from standard GSH solution determined by Saville's Method.

intracellular thiol depletion at 2 or 3 days (Fig. 3A). Depletion of cellular thiol by different BSO concentration was as in Fig. 3B and was significant even after a small amount of BSO. With the higher BSO concentration, there was more intracellular thiol depletion. The recovery of intracellular thiol concentration began within 2 days in BSO free medium and was 44% of normal concentration after 3 days (Fig. 3C).

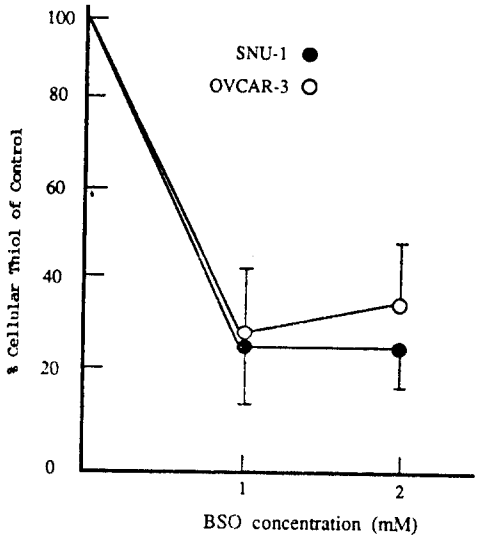

Fig. 2. Depletion of Cellular Thiol by BSO Treatment for 2 days in SNU-1 and OVCAR-3.

3. Effects of BSO on tumor cell growth and cytotoxicity

BSO inhibited partially the growth of SNU-1 and OVCAR-3 (Fig. 4). The growth inhibition effect of BSO was more significant with BSO 2 mM in OVCAR-3. SNU-1 was very sensitive to cisplatin but relatively insensitive to carboplatin. OVCAR-3 was relatively insenstive both to cisplatin and carboplatin. The cytotoxicities of cisplatin and carboplatin were markedly enhanced both in SNU-1 and OVCAR-3 by BSO treatment (Fig. 5). The enhancing effect was noted also with BSO 1 day treatment. There was a more enhancing effect with higher BSO concentration. SNU-1 and OVCAR-3 were relatively insensitive to radiation. The cytotoxicity of radiation (30 Gy) was increased significantly in OVCAR-3 (Fig. 6). In SNU-1, BSO effect on cytotoxicity of radiation was not noted with 1 mM but cytotoxicity was sligtly increased with BSO 2 mM.

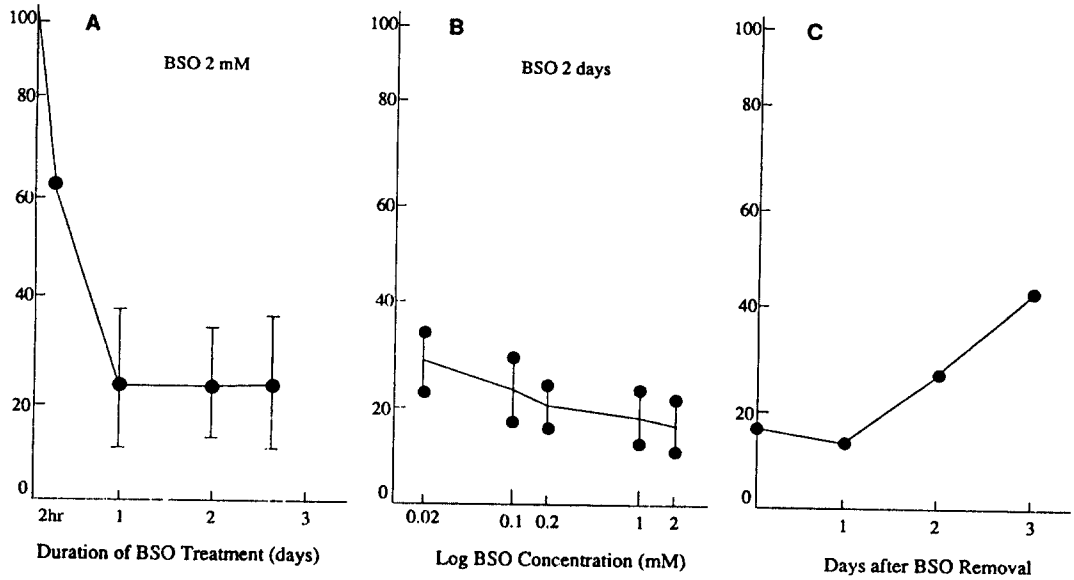

Fig. 3. Depletion of Cellular Thiol by BSO Treatment according to the Duration of Treatment (A) and BSO Concentration (B) Recovery of Cellular Thiol after Depletion by BSO Treatment (2 mM, 2 days) in SNU-1 (C).

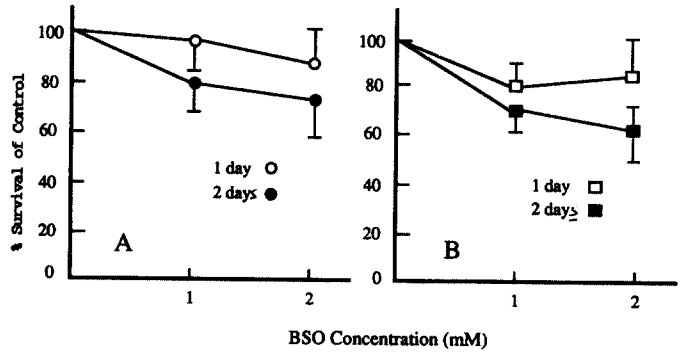

Fig. 4. Effects of BSO Treatment on Growth of SNU-1 (A) and OVCAR-3 (B)

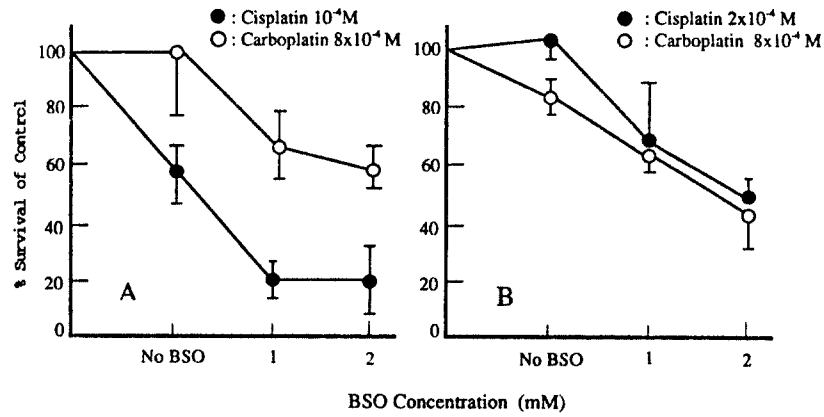

Fig. 5. Effects of BSO Treatment (2 days) on Cisplatin and Carboplatin Cytotoxicity in SNU-1 (A) and OVCAR-3 (B).

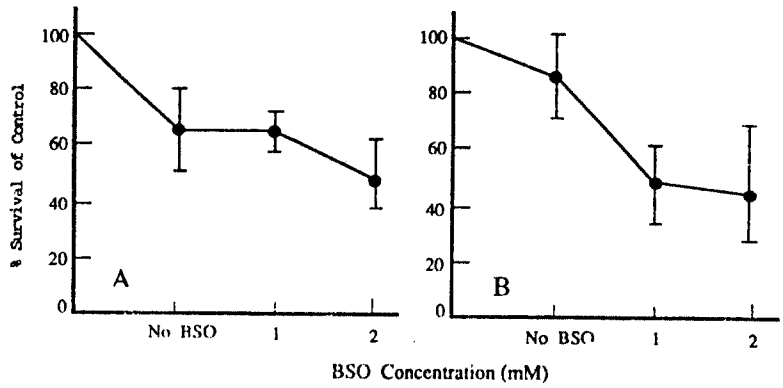

Fig. 6. Effects of BSO Treatment (2 days) on Radiation Cytotoxicity (30 Gy) in SNU-1 (A) and OVCAR-3 (B).

DISCUSSION

GSH is the major component of non-protein thiol (NPSH) and functions in synthesis of protein and nucleic acid, metabolism, enzyme activation, transport and cellular protection in normal cells[10]. Mainly through nucleophilic thioether formation or oxidation-reduction reactions, glutathione plays a role in the detoxification and repair of cellular injury by irradiation and antineoplastic agents such as melphalan and cyclophosphamide[11].

It has been reported recently that a relationship exists between intracellular glutathione levels and cytotoxicity to melphalan, cisplatin and irradiation in human ovarian cancer cell lines, and cell lines with resistance induced in vitro to either melphalan or cisplatin have a 2- to 3-fold elevation in intracellular GSH levels compared to the sensitive cell lines from which they were derived. Suzukake at al and Russo et al tried to overcome resistance and to enhance cytotoxicity by antineoplastic agents and irradiation by depletion of intracellular glutathione level[12,13].

Among thiol depleting agents including BSO, diamide, N-ethylmaleimide, dimethylfumarate and diethyl maleate, BSO depletes most efficiently intracellular thiol by inhibiting gamma glutamyl cysteine synthetase[14].

Our data showed that BSO effectively depleted intracellular thiol concentration both in SNU-1 and OVCAR-3. BSO 1 mM and 2 mM treatment for 2 days depleted intracellular thiol concentration 75.7% and 76.2% in SNU-1, and 74.1% and 63.0% in OVCAR-3, respectively. In melphalan-resistant human plastmacytoma, BSO 1-50 uM caused 80-90% depletion of cellular GSH[15]. In melphalan-resistant human stomach cancer cells[16], BSO reversed the expression of resistance to melphalan by inducing a 60% reduction in intracellular GSH content. In CHO cell lines, BSO depleted 90% of GSH and 80% of NPSH[17].

We observed that intracellular thiol concentration was depleted 33.4% at 2 hours BSO treatment. The intracellular thiol concentration was depleted 71.5% with BSO 0.02 mM treatment for 2 days in SNU-1 in this study and was depleted more with higher BSO concentration. Clark et al[17] observed that cellular GSH concentration was depleted 40% within 3 hours of BSO treatment and 65% with 1.0 uM BSO treatment. These findings suggest that BSO inhibits cellular GSH synthesis very early at low concentration.

In this study, the authors observed BSO inhibited tumor cell growth. The growth inhibition was remarkable with high BSO concentration (2 mM) and prolonged treatment (2 days). Clark et al[17] observed that BSO treatment with low concentration and short duration did not inhibit cell growth but more than 2 mM BSO treatment with longer duration significantly inhibited cell growth. Thus, it should be considered that GSH depletion by BSO may increase the side-effects of antineoplastic agents or irradiation.

Although the mechanism of cisplation resistance is still unclear, glutathione which inhibits the reaction between DNA and cisplatin (DNA cross-link) and DNA repair play big roles[18]. Glutathione can also react with monofunctional adducts in DNA to produce a glutathione-cisplatin-deoxyguanosine cross-link which would reduce the potential toxicity of the drug. Cellular GSH levels have been reported to be closely related to cisplatin cytotoxicity and cisplatin resistance. GSH was elevated in cisplatin resistant human tumor cell lines and BSO-induced GSH-depletion reduced the resistance for cisplatin and carboplatin[19]. Carboplatin is a cisplatin derivative without nephrotoxicity, and mode of action and antitumor effects are almost similar to cisplatin. The mechanism of resistance for carboplatin is still unknown. We observed that BSO enhanced not only cisplatin but also carboplatin cytotoxicity.

BSO is an effective radiosensitizing agent by thiol-depletion in hypoxic and in some oxic cells and increased hypoxic cell radiosensitization is inversely related to GSH concentration[17,20]. In this study, radiation cytotoxicities in SNU-1 and OVCAR-3 were increased by BSO induced thiol-depletion.

The authors observed that BSO could inhibit effectively the intracellular thiol concentration, and thus enhance the cytotoxicity of cisplatin, carboplatin and radiation. If BSO could be given to patients safely, it will enhance the treatment effect of antineoplastic agents and survival of the cancer patients. Because GSH depletion by BSO may increase the side effects of antineoplastic agents or irradiation, optimal dose, duration of treatment and treatment method of BSO should be evaluated appropriately before human use.

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