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Differential cytotoxicity of buthionine sulfoximine to "normal" and transformed human lung fibroblast cells

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Abstract. Glutathione (GSH) depletion has been studied extensively as a possible means to sensitize tumor cells to radiation treatment and chemotherapy. The present study was undertaken to compare the cytotoxicity of GSH depletion in normal and transformed cells. The results showed that specific inhibition of GSH synthesis by L-buthionine sulfoximine (BSO) caused significantly higher cytotoxicity in "normal" human-lung fibroblast cells than in their transformed counterparts. This finding suggests a possibility that depletion of GSH could be more harmful to normal cells than to transformed and/or tumor cells and that the selective cytotoxicity of BSO to normal cells could limit its potential as an effective sensitizer for cancer treatment.

Introduction

Glutathione (GSH) is a key component of the GSH-based detoxification system that protects cells against cytotoxicity and oxidative stress induced by radiation, therapeutic drugs, xenobiotics, and environmental toxicants [4, 18, 21, 29]. It plays an essential role in removing hydrogen peroxides and lipid peroxides by providing a substrate for GSH peroxidases (GPXs) [18]. It is also a scavenger of hydroxyl radicals, organic radicals, and peroxyl radicals [6]. Furthermore, GSH is a co-substrate for GSH-S-transferase that catalyzes the interaction between GSH and various xenobiotic compounds [18]. With some exceptions, tumor tissues and transformed cells generally have higher GSH contents than their normal counterparts [20, 25, 26]. In several experimental tumor models, elevated levels of cellular GSH and/or GSH-related enzymes have been shown to be associated with the resistance of tumor cells to radiation and therapeutic drugs [3, 27].

The intracellular GSH level can be modulated by L-buthionine sulfoximine (BSO), *N*-ethylmaleimide (NEM), diazenedicarboxylic acid bis(*N*,*N*'-dimethylamide) (diamide), and several other chemicals. BSO depletes cellular GSH by inhibiting gamma-glutamylcysteine synthetase, a key enzyme involved in the biosynthesis of GSH [13]. NEM is an alkylating agent that forms a covalent bond with thiols and irreversibly blocks the sulfhydryl groups [12]. Diamide oxidizes GSH into glutathione disulfide (GSSG) [15], which is recycled back to GSH by GSH reductase at the expense of reduced nicotinamide adenine dinucleotide phosphate (NADPH) [14, 18]. NEM and diamide react with GSH as well as protein thiols [12, 14]. Depletion of GSH by BSO alone or in combination with other thiol-reactive agents has been shown to sensitize tumor cells to cytotoxicities induced by radiation and many therapeutic drugs [5, 11, 16, 24, 28]. Treatment with BSO has also been found to reverse partially the multidrug resistance in cultured human breast tumor cells [10]. On the other hand, depletion of GSH by BSO has been shown to reduce the cytotoxicity of neocarzinostatin that undergoes bioactivation in vivo using GSH as a source of sulfhydryl groups [9]. These findings suggest that BSO could be useful for alteration of the cytotoxic effects of radiation and chemotherapeutic agents so as to improve the efficacy of cancer treatment and/or minimize the damage to normal tissues. Currently, BSO is under clinical evaluation as a potential sensitizer for cancer treatment with radiation and chemotherapy [23].

Treatment with BSO at concentrations ranging from 25 μ*M* to as high as 10 m*M* has been shown to induce minimal cytotoxicity in tumor cell lines with high cellular GSH levels, such as human adenocarcinoma cell line A549 [25], breast-tumor cell line MCF-7/ARD [10], cervical-carcinoma cell line HeLa S3 [25], and murine L1210 leukemia cells [16]. However, this conclusion may not apply to certain normal cells and/or tissues with low GSH contents.

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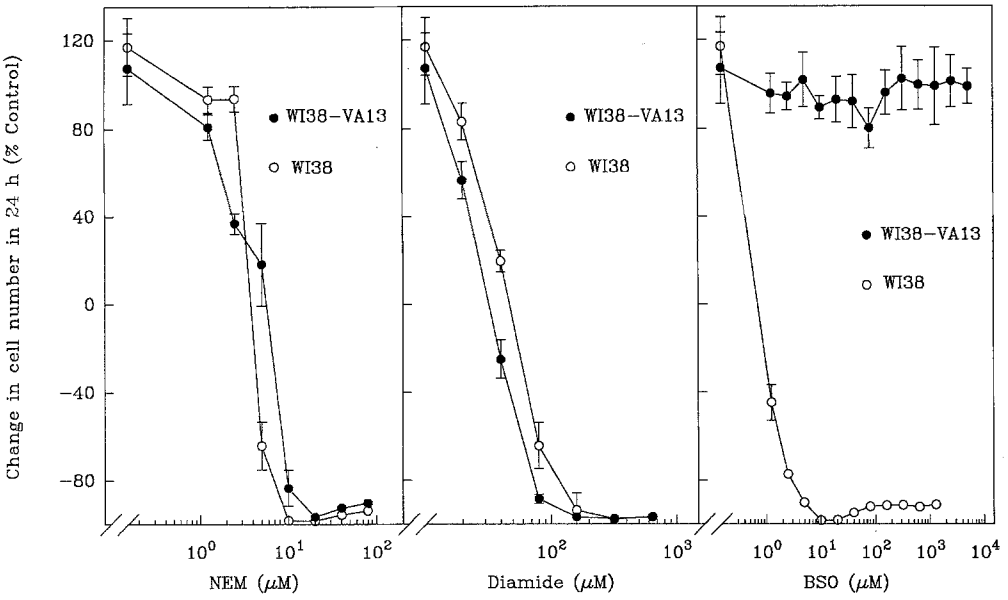

Fig. 1. Effect of NEM, diamide, or BSO treatment on the survival of WI38 and WI38-VA13 cells. The exponentially growing cells were incubated in BME medium containing 1.25–80 μ*M* NEM, 25–200 μ*M* diamide, or 1.25 μ*M* to 5 m*M* BSO for 24 h. After the incubation, the cells were washed with PBS, treated with 0.1% trypsin, and counted. The control cells were incubated in the medium without NEM, diamide, or BSO. Each data point represents the average (mean ± SD) of at least four measurements

The effect of BSO and other thiol-modulating agents on the growth and survival of normal cells and/or tissues has not been extensively studied. Such information is essential for a complete evaluation of thiol modulation as a means for potentiation of cancer radiation treatment and chemotherapy. The present investigation was undertaken to compare the effects of NEM, diamide, and BSO on the cellular GSH levels, the cell growth and survival of a "normal" human-lung fibroblast cell line, WI38, and its virally transformed subline, WI38-VA13. The results are discussed with respect to the differential growth inhibition and cytotoxicity associated with GSH modulation in normal and transformed cells.

Materials and methods

Cell lines and cell culture. The human-lung fibroblast line WI38 (ATCC CCL-75) and its SV40-transformed subline WI38-VA13 (ATCC CCL-75.1) were purchased from American Type Culture Collection. The two cell lines were maintained in Eagle's basal medium (BME) supplemented with 10% fetal bovine serum, 200 μ*M* L-glutamine, 100 units of penicillin sodium/ml, and 100 μg of streptomycin sulfate/ml (Gibco). The cells were kept at 37° C in a humidified atmosphere containing 5% CO_2 and 95% air and were subcultured weekly by treatment with trypsin. The WI38 cells used were from passages 16–19 and the WI38-VA13 cells were from passages 279–294.

Treatment with NEM, diamide, and BSO. To determine the effects of NEM, diamide, and BSO on the growth and survival of WI38 and WI38-VA 13 cells, the cells were plated on 24-well tissue-culture plates (Corning) at 4×10^3 cells/well and allowed to attach and grow for 72 h under the conditions described above. The cells were further incubated for 24 h in fresh BME medium containing 1.25–80 μ*M* NEM (Sigma), 20–640 μ*M* diamide, or 1.25 μ*M* to 5 m*M* BSO (Aldrich). Control cells were incubated in the medium without NEM, diamide, or BSO. After incubation, the growth medium was removed from each well and centrifuged at 3,000 rpm for 5 min, and the pellet was resuspended in 0.1 ml phosphate buffered saline (PBS) for microscopic examination. The cells remaining attached to the well were washed twice with PBS, treated with trypsin, and counted. Each experiment was conducted in quadruplicate. The cells used for the experiment were in the exponential growth phase. The viability of the control cells and the cells treated with NEM, diamide or BSO were determined by the dye-exclusion method. More than 90% of the cells attached to the wells after treatment were found to be viable, whereas few of the cells recovered from the growth medium were viable. For this reason, the number of cells remaining attached to the wells after the treatment was taken as the measurement of the viable cells.

To prepare cells for GSH measurement, WI38 and VA13 cells were plated in 150-mm tissue-culture dishes (Falcon) at 10^6 cells/dish and cultured under the conditions described above. When the cells had reached approximately 30%–50% confluence (in approximately 72 h), fresh media containing NEM, diamide, or BSO at various concentrations were added to the dishes, and the cells were further incubated in these media for 12 h. Control cells were incubated in BME medium without NEM, diamide, or BSO. After incubation, the cells were washed twice with cold PBS, harvested with a cell scraper, and centrifuged at 3,000 rpm for 5 min at 40° C. The cell pellets were resuspended in 3 vol. of 50 m*M* phosphate buffer (pH 7.8), frozen at –20° C overnight, and sonicated with a Fisher 550 sonic dismembrator with 3 bursts of 30 s each. The cell samples were stored at –20° C before the assay.

The protein concentrations of the samples were determined by a microassay method using the Bio-Rad protein-assay reagent [7], with bovine serum albumin (Sigma) serving as a standard.

Determination of GSH concentration. The levels of total cellular GSH were determined by Tietze's method [30] as modified by Bump et al. [8] using reduced GSH (Boehringer Mannheim) as a standard. The values obtained for the cellular GSH levels of the control cells and the cells treated with NEM, diamide, or BSO were analyzed by the analysis of variance and the Scheffe test.

Results

The effect of NEM, diamide, and BSO on cell growth and survival was compared in a "normal" human-lung fibroblast line, WI38, and its SV40 transformed subline, WI38-VA13. During the 24-h period of treatment, the numbers of

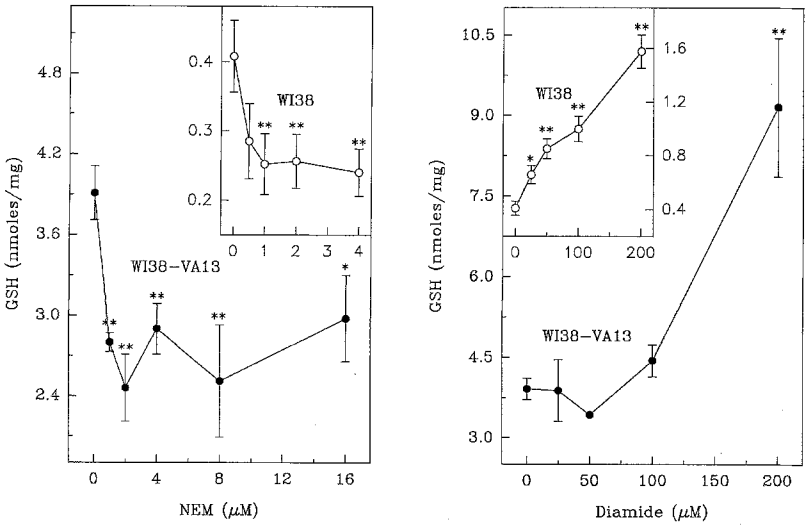

Fig. 2. Effect of NEM or diamide treatment on the GSH level of WI38 and WI38-VA13 cells. The cells were incubated in BME medium containing 0.5–16 μ*M* NEM (0.5–4 μ*M* for WI38 cells and 1–16 μ*M* for WI38-VA13 cells) or 25–200 μ*M* diamide for 24 h. Cells incubated in the medium without NEM or diamide were used as a control. The GSH levels were measured by the GSH reductase cycling method. Each data point represents the average (mean ± SD) of at least three measurements. The points marked with *one asterisk* and *two asterisks* are statistically different from the control at $P<0.05$ and $P<0.01$ levels, respectively

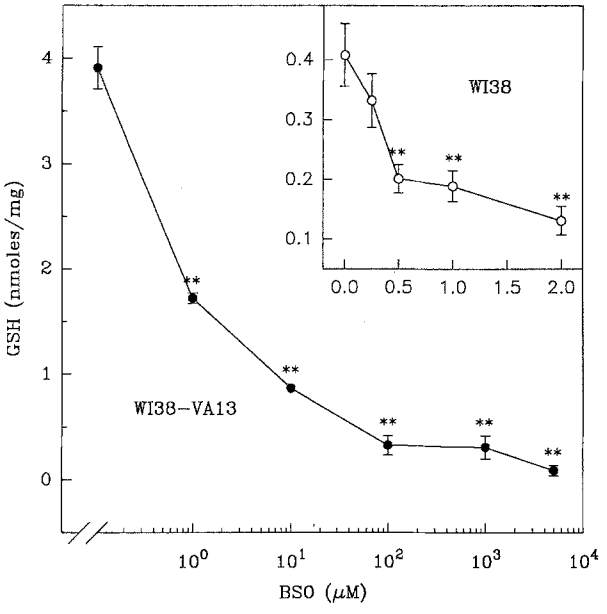

Fig. 3. Effect of BSO treatment on the GSH levels of WI38 and WI38-VA13 cells. The cells were incubated in BME medium containing 0.25–2 μ*M* BSO (WI38 cells) or 1 μ*M* to 5 mM BSO (WI38-VA13 cells) for 12 h. Cells incubated in the medium without BSO were used as a control. See the legend to Fig. 2 for a description of the method and data. The GSH levels of the WI38 cells treated with 2 μ*M* BSO and the WI38-VA13 cells treated with 5 m*M* BSO were 0.13 and 0.09 nmol/mg, respectively

WI38 and WI38-VA13 cells in the controls increased by 117% and 107%, respectively. Treatment with 1.25–80 μ*M* NEM or 20–160 μ*M* diamide caused dose-dependent growth inhibition and cytotoxicity in both cell lines (Fig. 1). The levels of growth inhibition and cytotoxicity induced by NEM and diamide in WI38 cells were close to the ranges induced by the two agents in WI38-VA13 cells, although WI38 cells appeared to be slightly more resistant to diamide treatment. In contrast, treatment with BSO induced significant growth inhibition and cytotoxicity only in WI38 cells (Fig. 1). Incubation of WI38 cells for 24 h in the medium containing BSO at a concentration of 2.5 μ*M* or higher decreased the number of surviving cells by approximately 80% or more. Similar treatment with BSO at concentrations as high as 5 m*M* not only failed to induce an appreciable level of cytotoxicity in WI38-VA13 cells but also failed to inhibit the growth of WI38-VA13 cells to a significant degree. Thus, treatment with BSO was apparently very toxic to WI38 cells but not to WI38-VA13 cells.

The cellular GSH levels of the untreated WI38 and WI38-VA13 cells were estimated to be 0.41 and 3.91 nmol/mg, respectively. Treatment with NEM caused a moderate decrease in the cellular GSH levels of both cell lines (Fig. 2). Incubation of WI38 cells for 12 h in the medium containing 0.5–4.0 μ*M* NEM reduced cellular GSH levels by 30%–41%. A similar treatment of WI38-VA13 cells with 1–16 μ*M* NEM decreased the levels of cellular GSH by 28%–37%. Treatment with diamide significantly increased the GSH levels of the two cell lines. Incubation for 12 h in the medium containing 25–200 μ*M* diamide increased the levels of cellular GSH by 61%–287% in WI38 cells and by 0.8%–134% in WI38-VA13 cells. The levels of growth inhibition and cytotoxicity caused by NEM and diamide did not correlate with the effects of the two agents on the cellular GSH level. Therefore, the growth inhibition and cytotoxicity induced by NEM and diamide might not result directly from the alteration of the cellular GSH level.

Treatment with BSO significantly reduced the levels of GSH in both WI38 and WI38-VA13 cells (Fig. 3). After a 12-h incubation in the medium containing 0.25, 0.5, 1, or 2 μ*M* BSO, the GSH level of WI38 cells decreased from 0.41 nmol/mg (control) to 0.33 (19%), 0.20 (51%), 0.19 (54%), and 0.13 (68%) nmol/mg, respectively. The level of GSH in WI38-VA13 cells decreased from 3.91 nmol/mg (control) to 1.72 (56%), 0.87 (78%), 0.33 (92%), 0.31 (92%), and 0.09 (98%) nmol/mg after a 12-h treatment with 1, 10, 100, 1,000, and 5,000 μ*M* BSO, respectively. A higher concentration of BSO was needed to decrease the GSH level in WI38-VA13 cells as compared with WI38 cells; however, in both relative and absolute terms the

cellular GSH was depleted to a greater extent in WI38-VA13 cells by 5 m*M* BSO than in WI38 cells by 2 μ*M* BSO. Thus, WI38-VA13 cells were apparently capable of sustaining GSH depletion to a significantly greater extent than were WI38 cells without displaying appreciable growth inhibition or cytotoxicity.

Discussion

Depletion of cellular GSH has been studied extensively as a possible means to sensitize tumor cells to radiation and many chemotherapeutic agents. However, the relative toxicity of GSH modulation to normal and transformed cells has not been investigated extensively. In the present study we compared the growth inhibition and cytotoxicity associated with GSH modulation in normal and transformed cells using cultured human-lung fibroblast cells as a model system. Treatment with either NEM or diamide caused comparable levels of growth inhibition and cytotoxicity in the "normal" human fibroblast cell line WI38 and its SV40-transformed subline WI38-VA13. The growth inhibition and cytotoxicity caused by NEM and diamide may not be directly related to the effects of these two agents on cellular GSH, since NEM reduced cellular GSH levels but diamide increased cellular GSH levels in both cell lines. The increased cellular GSH levels observed after diamide treatment could have resulted from an increase in GSH synthesis. The stimulation of GSH synthesis by diamide has previously been demonstrated in the housefly *Musca domestica* [1].

In contrast to NEM and diamide, BSO caused significant growth inhibition and cytotoxicity in WI38 cells but not in WI38-VA13 cells. It has been shown that BSO is only minimally toxic to transformed and/or cancerous cell lines [10, 16, 25]. In agreement with those studies, we found that treatment with BSO at concentrations as high as 5 m*M* did not significantly reduce the number of surviving WI38-VA13 cells as compared with the control. However, our results also demonstrated that BSO was highly toxic to WI38 cells. A 24-h incubation in the medium containing 2.5 μ*M* BSO decreased the number of surviving WI38 cells by more than 80%. These results suggest that treatment with BSO could be more toxic to normal cells than to transformed and/or cancerous cells. Two recent in vivo studies have shown that treatment with BSO is lethal to newborn rats [17, 22]. These findings indicate a possibility that depletion of GSH is highly toxic to certain normal cells and/or tissues.

The cause for the differential cytotoxicity induced by BSO in WI38 and WI38-VA13 cells is not clear, however, some intrinsic differences between the two cell lines could be contributing factors. One of the possibilities is that WI38-VA13 cells may need less GSH for survival than WI38 cells. This possibility is supported by the observation that a near-complete depletion of GSH in the WI38-VA13 cells treated with 5 m*M* BSO was not associated with any significant growth inhibition or cytotoxicity, whereas a moderate decrease in cellular GSH in the WI38 cells treated with 2 μ*M* BSO was accompanied by significant growth inhibition and cytotoxicity. We have also observed that the growth of WI38-VA13 cells was not inhibited by treatment with BSO at concentrations as high as 10–20 m*M* (unpublished observation). These findings suggest that some cellular process(es) in which GSH participates may be critical to "normal" human-lung fibroblast cells but not to their transformed counterparts. One of the important functions of GSH is to protect cells and mitochondria from damage induced by free radicals generated during oxidative metabolism [18, 19]. In comparison with normal cells, transformed cells generally depend less on aerobic respiration and more on glycolysis for their energy supply [2, 32]. If WI38-VA13 cells also have a high glycolysis-to-respiration ratio, like most transformed cells, they may not need much GSH to remove the reactive oxygen species generated during respiration.

Alternatively, WI38-VA13 cells may have a high level of other thiols in addition to GSH, and the other thiols may protect the cells when the GSH has been depleted. This hypothesis is consistent with the observation that the two nonspecific thiol-modulating agents NEM and diamide caused comparable levels of cytotoxicity in WI38 and WI38-VA13 cells, whereas the specific GSH-inhibiting agent BSO induced drastically different levels of cytotoxicity in the two cell lines (Fig. 1).

The differential cytotoxicity induced by BSO in WI38 and WI38-VA13 cells could also occur if the treatment with BSO were to impose a higher degree of oxidative stress on WI38 cells than on WI38-VA13 cells. We have recently demonstrated that treatment of WI38 cells with NEM, diamide, or BSO increases the activity of manganese superoxide dismutase (MnSOD) [31], an important mitochondrial enzyme that protects cells against oxygen free radicals. Similar treatment with NEM or BSO did not increase the MnSOD activity in WI38-VA13 cells. Treatment with diamide only marginally increased the MnSOD activity in WI38-VA13 cells (data not shown). Since MnSOD activity is generally increased under oxidative stress (see [33] for a list of agents that induce MnSOD), these findings support the notion that treatment with BSO may not impose a significant degree of oxidative stress on WI38-VA13 cells.

MnSOD protects cells by rapidly converting superoxide radicals to hydrogen peroxide, which is removed by catalase (CAT) and GPXs. Depletion of GSH by BSO could impair the function of GPXs, which use GSH as a co-substrate, thereby diminishing the capacity of the cells to remove hydrogen peroxide. WI38 cells contain significantly higher levels of MnSOD activity than do WI38-VA13 cells. A further increase in these high levels of MnSOD activity could overwhelm the cells with hydrogen peroxide. The combination of increased production of hydrogen peroxide and diminished function of GPXs could have indirectly contributed to the cytotoxicity observed in WI38 cells. It remains to be determined exactly which of these mechanisms, if any, might be responsible for the differential cytotoxicity induced by BSO in "normal" and transformed human-lung fibroblast cells.

Although the precise mechanism underlying the differential growth inhibition and cytotoxicity induced by BSO in "normal" and transformed human-lung fibroblast cells remains to be elucidated, our results suggest that treatment

with BSO could be more harmful to certain normal cells than to transformed and/or cancer cells. Although normal tissues as a whole might not be as sensitive to BSO treatment as the cultured WI38 cells, some subpopulation of the cells in normal tissues could be very sensitive to BSO treatment [17]. Further studies on BSO-induced cytotoxicity in normal and tumor tissues are needed to ensure the proper and effective use of BSO as a radio- and chemosensitizer.

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