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ABSTRACT

We have measured glutathione content in small tissue samples derived from biopsies of primary and metastatic human colon tumors and from colon cancer cell lines in tissue culture and xenografts in athymic mice. Measurements were performed using an enzymatic cycling assay designed to quantitate extremely low levels of glutathione (GSH) (down to 10^-14 mol) from perchloric extracts of tissue samples weighing less than 1 mg wet weight. Glutathione was stable in these acid extracts for at least 6 months when stored at -80°C. A survey of normal tissues in mice, rats, and some human tissues showed considerable variation in GSH content of different tissues but generally similar levels were identifiable for the same tissues from different species. The highest GSH level was 56.9 nmol/mg protein in rat liver and the lowest was 1.8 nmol/mg protein in rat skeletal muscle. High GSH levels were also determined in mouse and human liver, while low GSH levels were detected in mouse muscle. Human colon cancer cell lines showed slightly higher GSH levels than did colon cancer tumor samples obtained from biopsies. These studies revealed a marked interindividual difference in tumor GSH content, as well as a difference in GSH content between tumor deposits at different metastatic sites in the same individual. These results indicate the importance of direct tumor measurements of GSH content in clinical trials designed to modulate tumor glutathione content to try to increase sensitivity to chemotherapy or radiation therapy. Buthionine sulfoximine, an inhibitor of γ-glutamyl cysteine synthetase, was shown to produce almost complete depletion of GSH in four different human colon cancer cell lines in 24 h. Buthionine sulfoximine was also shown to be capable of producing drastic depletion of GSH in human colon cancer grown as xenografts in athymic animals.

INTRODUCTION

GSH is a naturally occurring tripeptide involved in a variety of biochemical and biological processes (1). As the major intracellular nonprotein thiol, it serves an important role in antioxidant defense, it scavenges free radicals produced by oxidation or radiation, and it serves as a nucleophilic sink for covalent adduct formation induced by a number of electrophilic agents (1). As a result of these processes, GSH protects cells against a variety of endogenous and exogenous toxic agents. Glutathione plays an important role in modulating the effect of chemotherapy and radiation therapy, and alterations in its concentration can drastically affect the response of tumor and normal tissues to these therapeutic interventions.

Elevated intracellular glutathione levels have been shown to be associated with melphalan resistance in a number of cell lines including murine L1210 leukemia (2), human plasmacytoma (3), gastric carcinoma (4), ovarian carcinoma (5), and rhabdomyosarcoma (6). Conversely, agents that lower GSH content can sensitize cells to alkylating and platinating agents and radiation therapy (5–13). Alkylating agent sensitization produced by GSH depletion has been demonstrated in model systems for human ovarian cancer (5, 7, 8), human lung cancer cell lines (9), and human medulloblastoma and glioma cell lines growing as xenografts in athymic mice (10). These studies suggest that depletion of GSH may provide an approach to sensitize tumors to chemotherapy or radiation therapy. A potentially useful agent for this approach is BSO, which functions as a highly specific inhibitor of γ-glutamyl cysteine synthetase, the first enzyme in the glutathione synthesis pathway (14). By inhibiting this essential enzyme, BSO has the capacity to drastically reduce glutathione content and has been shown in model systems to enhance the cytotoxic effects of specific chemotherapeutic agents and radiation therapy (5–13). BSO and other agents directed at modulating glutathione levels affect the content of this metabolite in normal tissues as well as in tumors (2, 14).

In order to most effectively use a biochemical modulator to enhance chemotherapy or radiation therapy, it is important to be able to monitor the effect of the modulator on the target tumor tissue. This provides the information necessary to evaluate the efficacy of modulation and potential reasons for success or failure of a modulator-based regimen. For example, if a regimen using a biochemical modulator does not yield improved therapeutic results relative to the same regimen given without the modulator, it is important to determine whether the modulator failed to achieve the desired biochemical end point, in which case the modulator can be adjusted to achieve this end point. Alternatively, it is possible that the modulator did achieve the desired biochemical end point but this did not increase the efficacy of the primary therapeutic agent.

Because of problems with obtaining sufficient tumor tissue for biochemical analysis, the effects of modulators are frequently not measured at all or are measured on easily obtainable, surrogate tissue such as peripheral blood mononuclear cells (15, 16). However, these cells may not reflect the metabolic changes of the tumor; therefore, it is preferable to obtain direct biochemical measurements from the tumor. With the development of computed tomography-guided biopsy techniques for diagnostic purposes, virtually all intraabdominal and pelvic tumors have become accessible for needle biopsies. This approach may provide sufficient tissue for both histological and biochemical analysis, especially when enzymatic cycling techniques are used to amplify the reaction product (17).

To overcome the problems associated with biochemical measurements in very small tissue samples, we have used an enzymatic cycling assay to amplify the reaction product (17). By increasing the number of cycles used in the assay system, GSH can be measured in concentrations as low as 10^-14 mol. We have used this method to characterize the glutathione levels in normal and tumor tissue and to effectively monitor the ability of agents such as BSO to modulate glutathione levels. This method is rapid, reproducible, and capable of measuring a wide range of glutathione levels present in needle biopsies of tumors without the need for measurements in surrogate tissues.
The assay, based on the method of Tietze (18) and that of Brehe and Burch (19), is outlined below and was modified for application to perchloric acid extracts of very small tissue samples.

First Step

Second Step - Cycling Step

Third Step

As shown in the first step reaction, DTNB, designated as the sulfhydryl donor, RSSR, is reduced in stoichiometric proportion to the oxidation of glutathione in a NADP⁺-dependent reaction catalyzed by glutathione reductase. In the Tietze assay, the rate of reduction of DTNB provides the basis to quantitate glutathione when the latter is present in sufficient quantity. In the second step, to amplify NADP⁺ through production of 6PG in the NADPt dependent glucose-6-phosphate dehydrogenase (G6PDH) reaction (17, 19). The NADP⁺ generated in the first reaction was quantitated after stopping the reaction with HCl and heat. Aliquots were transferred to a cycling reagent system and the NADP⁺ was amplified by cycling through the reaction indicated in the second step, producing 6PG in proportion to the cycled NADP⁺. The second reaction was stopped with heat. The third and final reaction was initiated by addition of fresh 6-phosphogluconate dehydrogenase (6PGDH) and an excess of NADP⁺, which is quantitatively converted to NADPH in proportion to the 6PG formed in the second step. NADPH is determined fluorometrically and used to calculate the amount of GSH in the original extract relative to standards measured simultaneously with the same cycling reaction. This assay measures total glutathione, oxidized and reduced, and is therefore not affected by oxidation of the original sample. This assay is rapid, reproducible, and applicable to very small samples. By increasing the number of cycles used to amplify the NADP⁺ generated in the first reaction, we have been able to easily measure glutathione in less than 10⁴ cells obtained from tissue culture or in very small tissue fragments cut from needle biopsy specimens.

MATERIALS AND METHODS

DTNB was purchased from Aldrich Chemicals (Milwaukee, WI). Glucose-6-phosphate dehydrogenase (EC 1.1.1.43) was obtained from Boehringer Mannheim Biochemicals (Indianapolis, IN). Glutathione reductase from yeast (EC 1.6.4.2), NADPH, NADP⁺, GSH, l-buthionine-[S,R]-sulfoximine, other chemicals, and enzymes were obtained from Sigma Chemical Co. (St. Louis, MO).

Preparation of Tissue Extracts. Tissue extracts for glutathione analysis were prepared from human tissues obtained at surgery or from rodent tissues obtained from C57BL/6 mice, athymic mice, or Sprague-Dawley rats. Animals were anesthetized with i.v. pentothal, and then organs were rapidly excised and frozen in liquid nitrogen within 10–30 s after the blood supply was cut off. Samples weighing 10–30 mg were sliced, weighed in the frozen state in a −20°C cold room, and transferred into prefrrozen 10–75-mm glass tubes. The samples were allowed to thaw in 100 µl of 1.75% perchloric acid in an ice bath. Mixtures were homogenized with a glass rod and diluted to 0.35 N HClO₄ with ice-cold, deionized, distilled water and mixed well. Homogenates were kept on ice for 20 min and then centrifuged at 10,000 × g for 2 min at 4°C. Pellets were saved for protein determination (20). Four hundred µl of each supernatant were transferred to another 10–75-mm tube and neutralized to pH 7.0 by the addition of 50 µl 2.1 M KOH and Imidazole base-0.5 M KCl. Mixtures were then kept on ice for 15 min and centrifuged at 10,000 × g for 2 min at 4°C. Supernatant was removed and stored at −80°C. 0.5- to 2-µl aliquots were used to measure total glutathione. When stored at −80°C, total reduced and oxidized GSH content in these neutralized extracts, as well as in standards, was extremely stable, even at low concentrations.

Preparation of Extracts from Needle Biopsies. Needle biopsies of tumor samples were obtained during surgery and immediately frozen in liquid nitrogen within 15 s. Sections (usually <1 mg) were cut from the biopsy at −20°C and placed in Eppendorf centrifuge tubes; adjacent sections were taken for histology. The tissue was allowed to thaw in 30 µl of 1 N HClO₄ in an ice bath and then homogenized with a glass rod. The homogenate was diluted to 85 µl with ice-cold, deionized, distilled water to obtain a final concentration of 0.35 N HClO₄. The mixture was kept on ice for 20 min and then centrifuged at 10,000 × g for 2 min at 4°C. The pellet was saved for protein determination (20). Eighty µl of supernatant were transferred to another Eppendorf tube and neutralized to pH 7 with 10 µl of 2.1 N KOH and 0.5 M KCl as described above. The mixture was centrifuged at 10,000 × g for 2 min at 4°C in a microcentrifuge. Supernatants were removed and used to measure glutathione or stored at −80°C until measured. The remainder of the procedure was carried out as described above for normal tissues.

Preparation of Extracts from Cells in Tissue Culture. For preparation of extracts from cells growing in tissue culture, cells were harvested by aspiration and collected by centrifugation at 3000 × g for 3 min. Medium was removed by aspiration and acid extracts were prepared by adding 110 µl 0.35 N HClO₄ to cell pellets, which were mixed well and incubated in an ice bath for 15 min. Extracts (80 µl) were neutralized to pH 7.0 with 2.1 N KOH, 1 M Imid base, and 0.5 M KCl. The mixture was kept on ice for 15 min and then centrifuged and processed as indicated above for measurement of glutathione and protein content.

Tissue Culture Procedure. Tissue culture and xenograft procedures for the Case Western Reserve University Colon Cancer Cell Bank have been described previously (21–23). Human colon cancer cell lines HCT116, VACO 5, VACO 6, and VACO 8 were grown at 37°C in plastic substrates in minimum essential medium with Earle’s salts supplemented with 0.1 mM nonessential amino acids, gentamicin (25 µg/ml), and 8% heat-inactivated Hyclone calf serum. Cells were dissociated and passed as described previously (21–23). For experiments with BSO, VACO 6 and VACO 5 were replated in 24 ml medium at 10⁶ cells/ml in T-75 flasks. VACO 8 and HCT116 were set to grow at 2 × 10⁶ cells/well in 6-well trays. Cells were incubated at 37°C overnight and the various concentrations of BSO were added, while control cells were incubated without added BSO. At each time point, cells were removed from flasks or dishes and collected by centrifugation at 4°C for 3 min at 3000 × g; perchlorate extracts were performed as indicated above.

Xenograft Establishment. VACO 6 human colon tumor cells were grown in tissue culture and injected at 5 × 10⁶ cell/0.2 ml minimum essential medium without serum into both anterior shoulders of female athymic BALB/c-nu/nu mice. When the xenografts reached volumes of approximately 150 mm³, animals were treated with BSO, 2.5 mg/kg i.p., at 0, 15, 24, and 39 h and were allowed to drink water containing 20 mM BSO ad libitum for 43 h. The control animals were given injections of saline and were allowed to drink water ad libitum. At the end of 43 h, animals were anesthetized and the xenografts, livers, and kidneys were removed within 5 to 10 seconds and frozen immediately in liquid nitrogen. The perchloric acid extracts of the tissues were prepared as explained above.

Glutathione Assays. All reagents were prepared fresh daily from stock solutions of each component as described by Tietze (18) and Brehe and Burch (19). Reagent I contained 100 mM NaHPO₄ (pH 7.0), 2 mM EDTA, 0.02% bovine serum albumin, and 0.2 µM DTNB. Reagent II contained 100 mM Imid-HCl (pH 7.0) (50 mM Imid base-50 mM Imid HCl), 1 mM EDTA, 0.02% bovine serum albumin, 0.4 mM NADPH, 30 µM ascorbic acid (prepared fresh), and 6 µg/ml glutathione reductase. NADP⁺ cycling reagent was prepared as described by Lowry and Passonneau (17) except for the levels of enzymes used. The reagent contained sufficient amounts of both glucose-6-phosphate dehydrogenase and glutamate dehydrogenase to give approximately.
2,000–20,000 cycles/h (17). The final indicator reagent was prepared as described by Chi et al. (24). ATP and NAD+ levels were measured by enzymatic cycling assays as described previously (17, 24–27). Protein was measured using the method of Lowry et al. (20) on the precipitates obtained from the perchloric acid extracts.

**Samples Containing 5 to 100 pmol of GSH.** Extracts or standards (0.5–1.0 μl) in neutralized perchlorate extract were added to 40 μl of reagent I in a 10- x 75-mm glass tube and placed in an ice bath. The tubes were mixed and then 40 μl of reagent II were added to each tube at timed intervals. The tubes were transferred from the ice bath to a water bath at 24°C for 10 min. The reactions were arrested by transferring the tubes into an ice bath and adding 20 μl of 0.25 n HCl at timed intervals so that reaction times were the same for each tube. All reaction tubes were then heated at 100°C for 3 min. The tubes were cooled and then 1 μl of reaction mixture was transferred to 100 μl 0.2 N HC1 at timed intervals so that reaction times were the same for each tube. After heating at 100°C for 3 min. After cooling, 1 ml of indicator reagent was added to each tube and mixed well. The fluorescence developed was read using a Fluoromax fluorometer at 365 nm excitation and 460 nm emission.

**Samples Containing Less Than 5 pmol (BS0-treated Tissues or Cells).** The volumes of reagents I and II were reduced to 15 μl and the reactions were conducted in 5- x 50-mm microtubes. One to 2 μl of HClO4 extract of tissue or standards (0.5 to 5 μM GSH) were added to reagent I. The incubation time was increased to 30 min. The reaction was stopped by the addition of 10 μl of 0.2 N HCl at timed intervals as explained above. After heating at 100°C for 3 min, the samples were cooled and 1-μl aliquots were transferred into 50 μl of cycling reagent. The cycling was increased to 15,000–20,000 cycles in 60 min.

**RESULTS**

In this article, we describe an enzymatic cycling assay capable of accurately and reproducibly measuring glutathione content in perchloric acid extracts prepared from very small samples of cells or tissues. Fig. 1A shows the standard curve for measuring GSH over a wide range of concentrations from 5 to 100 pmol using a reaction conducted for 10 min at 24°C. Lower concentrations can be accurately measured by increasing the reaction time and/or reducing the reaction volume. As shown in Fig. 1B, GSH concentrations of 1–10 pmol were more accurately measured by increasing the reaction time to 30 min. As shown in Fig. 1C, levels of 0.1–1.0 pmol were assayed by reducing the volume of reagents I and II to 15 μl each and incubating for 30 min. Fig. 1D shows that we have accurately measured GSH concentrations ranging from 0.01 to 0.1 pmol by extending the incubation time to 45 min. GSH in amounts less than 0.05 pmol can be measured in a reaction volume of 3–5 μl by conducting reactions under oil to prevent evaporation (17). In order to measure very low levels of GSH, special precautions need to be taken with the reagent blank. These precautions include the use of small aliquots of the reaction mixture at the cycling step and heating NADPH in 50 mM HCO3 buffer, pH 10.0, at 100°C for 5 min before use as a reagent.

**GSH Levels in Animal Tissues.** To determine the range of values that might be expected in different normal tissues, as well as to obtain an indication of the relative levels in different species, we used the methods described above to measure GSH in multiple organs from normal mouse and rat. Table 1 shows the values obtained on duplicate samples of HClO4 extracts from 2 to 4 different pieces weighing approximately 15–30 mg/tissue, wet weight. These results agree with relative values reported previously for GSH levels in rodent tissues (14). However, most earlier reports expressed GSH levels on a wet weight tissue basis, whereas in the present report GSH levels are expressed relative to protein content.

The highest levels of GSH were found in the liver of both mouse and rat, ranging from 39 to 57 nmol/mg protein. This is in marked contrast and is approximately an order of magnitude greater than the levels of GSH in muscle tissues from both species, 1.8–2.8 nmol/mg protein. Heart muscle in mice was higher than skeletal muscle but was relatively low compared to other organs. Heart muscle in rats was in the midrange for other rat tissues. Species differences also were notable in mouse brain stem and cerebellum, which contained 6–7 times higher GSH than those of rats, whereas the cerebral cortex exhibited the same range of values in both species. These studies demonstrate the wide variation of GSH levels in different tissues and the similarities, as well as some differences, in tissues from different species.

**GSH Levels in Human Tumors.** GSH levels were determined in a series of needle biopsies of human tumors obtained mostly from patients with adenocarcinoma of the colon. The needle biopsies were performed on January 5, 2018. © 1994 American Association for Cancer Research.
The GSH content of primary colon tumors ranged from 5.5 to 14.2 nmol/mg protein. Each section was subject to separate HClO4 extraction and analyzed for GSH as indicated in “Materials and Methods.” Table 2 shows the results of the GSH assays based on the location of the tumor biopsies. The GSH content of primary colon tumors ranged from 5.5 to 14.2 nmol/mg protein. The lowest value of 5.5 nmol/mg protein occurred in a primary tumor of the cecum. At the present time, data are insufficient to determine whether GSH content varies with the location of the primary tumor. The mean value for GSH content of primary colon adenocarcinoma is 11.1 nmol/mg protein. The single colon polyp analyzed in this study had a GSH content of 8.5 nmol/mg protein. The mean ± SEM obtained from duplicate assays on two samples from each biopsy are shown in Table 2.

In one case, we had the opportunity to measure GSH levels in several samples of normal and tumor tissue removed simultaneously from a single patient. Table 3 shows that the GSH level in the primary colon cancer metastatic to the liver was in the same range as normal liver. This case clearly demonstrates that different tumor deposits in the same patient can have highly variable GSH levels, which may be influenced by surrounding tissue.

**Effect of BSO on GSH in Colon Cancer Cell Lines.** Buthionine sulfoximine interferes with the first step in GSH synthesis by specifically inhibiting γ-glutamyl cysteine synthetase and thereby has the potential for depleting GSH levels (14). To evaluate the ability of BSO to effectively deplete GSH in human colon cancer, we used the enzymatic cycling assay to measure GSH content in BSO-treated tissue culture cells and xenografts in athymic animals. Cells in tissue culture were continuously exposed to selected concentrations of BSO. Samples were removed and HClO4 extracts were performed at the indicated time points, stored at −80°C, and then assayed when all samples from an experiment were collected. As shown in Fig. 2, exposure to 0.1 and 1 mM BSO resulted in significant depletion of GSH in all four cell lines. The pattern of GSH depletion, however, varied from cell line to cell line. The control levels of GSH at the beginning of the experiment varied also from 7.0 to 30.7 nmol/mg protein. The untreated HCT116 and VACO 5 cell lines showed an initial increase in GSH levels as the cells progressed into more rapid log phase growth during the first 48 h in culture. While the response to BSO varied among the four cell lines, they all showed a decrease in GSH within 3 h of treatment with 0.1 mM BSO. There was a rapid fall in GSH concentration in HCT116, whereas the fall in GSH was more gradual in the other three cell lines. Within 24 h, all the cell lines treated with 0.1 mM BSO showed 86–98% depletion of GSH relative to BSO varied among the four cell lines, they all showed a decrease in GSH within 3 h of treatment with 0.1 mM BSO. There was a rapid fall in GSH concentration in HCT116, whereas the fall in GSH was more gradual in the other three cell lines. Within 24 h, all the cell lines treated with 0.1 mM BSO showed 86–98% depletion of GSH relative to the observation of higher levels of GSH in mouse and rat livers as indicated in Table 1. GSH in colon cancer metastatic to the liver was in the same range as normal liver. This case clearly demonstrates that different tumor deposits in the same patient can have highly variable GSH levels, which may be influenced by surrounding tissue.
to simultaneous controls; even greater depletion occurred with 1 mM BSO at this early period. By 48 h, GSH was reduced to levels between 0.3 nmol/mg protein in HCT116 and 5.9 nmol/mg protein in VACO 6. Increasing the concentration of BSO to 1 mM showed a very small advantage in further depleting the GSH levels in the cell lines. The extended treatment of these four cell lines with 0.1 or 1 mM BSO exhibited no apparent cytotoxicity. Treatment with concentrations as low as 10 μM BSO produced 82% GSH depletion in VACO 5, 78% in VACO 6, and 45% in VACO 8 at 48 h (results not shown). These studies show the effectiveness of BSO in depleting GSH levels in colon cancer cell lines and the ability to reproducibly monitor GSH at very low levels using the currently described technique. They also illustrate the potential variability in GSH depletion and the need for monitoring to measure the biochemical effects of the modulator.

**Effect of BSO on GSH in Colon Cancer Xenografts.** The effect of BSO on GSH concentration in colon cancer in vivo was studied with VACO 6 xenografts established by injection of tissue culture cells into athymic mice. The glutathione concentration was measured in normal livers, kidneys, and tumors from control and BSO-treated mice. We also used enzymatic cycling assays to measure NAD⁺ and ATP in the same HClO₄ extracts of these tissues. Table 4 shows that the GSH concentrations in normal liver and kidney from tumor-bearing athymic mice were in the same range as those noted for the normal tissues from C57BL/6 mice as reported in Table 1. The tumor xenografts in untreated mice exhibited GSH values that were slightly lower than logaritically growing VACO 6 in tissue culture. Four i.p. injections of BSO, 2.5 nmol/kg, and administration of BSO, 20 mmol/liter for 43 h in drinking water, depleted GSH by more than 90% in xenografts as well as in other normal organs, including liver and kidney. Although BSO treatment had a drastic effect on GSH content, there were no apparent clinical signs of toxicity and BSO had negligible effects on the NAD⁺ or ATP content. Thus, under these conditions, BSO showed a lack of specificity for normal versus tumor tissue but was highly specific from the metabolic viewpoint.

**DISCUSSION**

The demonstration that GSH depletion sensitizes cells to the cytotoxic effects of several alkylating agents and radiation provides a strong rationale for developing cancer therapy regimens that combine alkylators or radiation with biochemical modulators to reduce GSH levels. Buthionine sulfoximine, an inhibitor of γ-glutamyl cysteine synthetase, is effective in depleting GSH and has already entered clinical trials (14–16). In most cases, the effects of BSO have been evaluated by monitoring GSH in peripheral blood mononuclear cells (15, 16). In a very limited number of cases, treatment of patients with BSO has shown to decrease GSH content in peripheral blood, mononuclear cells, and tumor cells obtained from ascitic fluid (15, 16). However, in at least one BSO-treated patient, peripheral blood mononuclear cells showed an 88% decrease in GSH content but no change in GSH occurred in tumor tissue (15). This case clearly illustrates the difficulty in trying to evaluate the effect of a metabolic modulator by analyzing surrogate tissues such as peripheral blood mononuclear cells. Since for most of the patients in the reported clinical trials GSH levels were measured only in peripheral blood mononuclear cells, it is not clear whether GSH was actually depleted in tumor tissues or how this may have affected the apparently low response rates (15, 16).

In order to effectively monitor changes in GSH content in tumor or normal tissue, we have used an enzymatic cycling assay which allows accurate measurements of GSH in extracts prepared from very small fractions of needle biopsy specimens. Since only a small piece of the biopsy specimen is required for chemical analysis, adjacent tissues can be submitted to microscopic examination for histological evaluation. We have used this approach to confirm the histological characteristics of the samples used for biochemical measurements. Thus, we have been able to confirm that biochemical measurements were actually performed on tumors or normal tissues.

The enzymatic cycling assays are rapid, reproducible, and inexpensive, and we have shown that levels of total GSH are stable in tissue extracts stored at −80°C for at least 30 days. By increasing the number of cycles in the assay system, we have measured GSH quantities as low as 10−14 mol in tissue and cell extracts.

Our studies show significant differences in GSH content of different mammalian tissues, with muscle having the lowest GSH and liver the highest. These observations suggest that each organ will have its own characteristic response to modulators such as BSO and that different effects on GSH content may alter the spectrum of toxic effects of chemotherapy or radiation therapy. The variation of GSH levels in different tissues supports the notion that accurate determination of the effect of modulators will require chemical analysis of specific normal or tumor tissues rather than surrogate samples.

Although GSH content is an important determinant of responsiveness to drugs and/or radiation, it should be remembered that this process is significantly affected by components of glutathione metabolism, including the enzymes γ-glutamyl cysteine synthetase, glutathione peroxidase, and glutathione S-transferase (28–33). This is especially true with respect to the radiation response where GSH depletion may not inhibit GSH peroxidase since bound sulfhydryls may be present in sufficient quantities to reduce hydroperoxides (34). Differences in GSH-metabolizing enzymes may also contribute to differential changes in drug or radiation sensitivity in tumor and tissues used for surrogate measurements.

Our measurements of GSH levels in multiple tumor biopsies demonstrate the feasibility of this approach for direct monitoring of GSH content. Thus, GSH can be measured by enzymatic cycling assays in almost any tissue that can be sampled by direct or computed tomography-guided needle biopsy. These studies show considerable GSH variation in different tumors, as well as in primary colon adenocarcinomas from different patients. There was also heterogeneity of GSH content in colon cancer metastatic to different sites with a tendency

### Table 4: Total GSH in normal and BSO-treated mice

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Control (nmol/mg protein)</th>
<th>BSO-treated (nmol/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>GSH</td>
<td>NAD⁺</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Xenograft</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Right</td>
<td>32.6 ± 1.5*</td>
<td>2.0 ± 0.04</td>
</tr>
<tr>
<td>Left</td>
<td>20.6 ± 2.6</td>
<td>1.59 ± 0.04</td>
</tr>
<tr>
<td>Normal</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Liver</td>
<td>45.5 ± 4.3</td>
<td>3.48 ± 0.29</td>
</tr>
<tr>
<td>Kidney</td>
<td>13.2 ± 0.8</td>
<td>2.5 ± 0.16</td>
</tr>
</tbody>
</table>

*Mean ± SEM of duplicate assays performed on each tissue from two control mice or three BSO-treated mice.
for metastatic deposits in the liver to show higher levels than those in lymph nodes. In one patient we observed a 2-fold difference between the GSH content of the primary colon carcinoma (14.2 nmol/mg protein) and a metastatic lesion in the liver (33.4 nmol/mg protein). It is possible that the heterogeneity of GSH levels in metastatic tissue is influenced by the location and metabolic activity of the surrounding tissues. These variations in GSH content may contribute to the heterogeneity of tumor responses to therapeutic agents and may also lead to the selection of resistant populations. In addition, the heterogeneity of GSH levels in different metastatic tumor deposits may affect the efficacy of metabolic modulators such as BSO.

The GSH levels in human colon cancer cells growing in tissue culture were in a similar range but slightly higher than those found in needle biopsies from human tissues. The cellular GSH content decreased when tissue culture cell lines were planted as xenografts in athymic mice. The difference between the GSH content in the tissue culture cells compared to those growing as xenografts in athymic mice may be due to higher oxygen concentration in tissue culture inducing higher levels of GSH as an antioxidant defense. It has previously been noted that cells in logarithmic growth have higher GSH levels than do those in plateau phase (35). Thus, the higher level of GSH in tissue culture may also reflect a higher growth fraction under these conditions. It is also possible that the different GSH levels observed in tumor metastasized to different sites may reflect site-dependent variation in tumor growth or metabolism.

These studies show that BSO is capable of depleting GSH in human colon cancer cell lines in tissue culture, as well as in xenografts in athymic mice. The effect of BSO in vivo is nonspecific on a tissue basis since we observed drastic reduction in GSH content of liver and kidney, as well as in tumor xenografts. It is, however, feasible that with lower doses or different schedules of BSO administration, it would be possible to more selectively lower GSH content of tumor relative to normal tissues. Although the glutathione-lowering effect of BSO was not selective on a tissue basis, it was relatively specific from a metabolic viewpoint since ATP and NAD were not affected in the presence of drastic depletion of GSH. Preservation of metabolites such as ATP and NAD may account for the apparent lack of toxicity associated with GSH depletion. However, the effects of GSH depletion are expected to be more apparent when animals are oxidized, alkylating agents, or radiation.

Previous studies have shown that some patients with advanced metastatic colon cancer undergo marked tumor regression following treatment with melphalan (36, 37). As noted above, BSO depletion of GSH has been shown in a variety of model systems to sensitize tumors to melphalan-based chemotherapy (3–8, 10). These observations, coupled with the ability to monitor GSH content in tumor biopsies, provide an indication that it would be useful to conduct a clinical trial with BSO plus melphalan for the treatment of colon cancer with metastatic deposits in the liver to show higher levels than those in plateau phase (35). Thus, the higher level of GSH in tissue culture cells compared to those growing as xenografts in athymic mice.

REFERENCES

Sensitive Enzymatic Cycling Assay for Glutathione: Measurements of Glutathione Content and Its Modulation by Buthionine Sulfoximine in Vivo and in Vitro in Human Colon Cancer

Sosamma J. Berger, David Gosky, Elizabeth Zborowska, et al.


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