ABSTRACT

Cellular glutathione (GSH) levels were measured from 27 human lung tumor biopsies, enzymatically disaggregated, and compared with cells isolated from normal lung of the same patients. GSH levels from normal lung were similar among patients with a mean value of 11.20 ± 0.58 (SEM) nmol GSH/mg protein (24 patients) with a range from 6.1 to 17.5 nmol GSH/mg protein. GSH levels varied considerably within and across histological tumor types with the following values: adenocarcinomas, 8.83 ± 0.96 nmol/mg protein (8 patients); large cell carcinomas, 8.52 ± 2.51 nmol/mg protein (3 patients); and squamous cell carcinomas, 23.25 ± 5.99 nmol/mg protein (8 patients). The cyclic GSH reductase assay gave only average GSH values and could not distinguish possible GSH variation among subpopulations of cells isolated. Cell volume measurements and microscopic evaluation of cells isolated from both tumors and normal lung revealed heterogeneity with respect to cell types present. To determine the extent of thiol variation among tumor cell subpopulations, tumor cell suspensions were stained with the thiol-specific stain, monochlorobimane (MCB). The accuracy of MCB staining was tested by flow cytometric analysis of 12 in vitro human tumor cell lines and 3 rodent cell lines. A linear relationship was found between the bimane cellular fluorescence and the cyclic GSH reductase assay for cell lines having less than 80 nmol GSH/mg protein ($R^2 = 0.82$). Above 80 nmol GSH/mg protein the rate of change of the bimane fluorescence intensity with respect to increasing GSH concentrations was much reduced. However, by labeling cells with MCB it was possible to distinguish between cell lines with low versus high GSH content. MCB staining of tumor samples revealed multiple populations of cells with respect to thiol levels. In particular, 2 of 8 squamous cell carcinomas had a proportion of cells with elevated fluorescence intensities (from 10 to 35% of the population) suggesting the presence of cells with greatly elevated thiol levels. These findings underscore the complexity of quantitating intracellular GSH levels from tumor biopsies. The combined use of MCB with flow cytometry and conventional GSH assays may help to delineate subpopulations of cells within tumors with different thiol levels.

INTRODUCTION

Drug resistance of tumors poses a significant problem in cancer treatment. The problem is further compounded by the fact that tumors may become resistant to diverse chemotherapy drugs with differing modes of cell killing. Generally, normal tissues fail to develop resistance to drug treatments thereby rendering the prospect for obtaining a therapeutic gain for treatment of solid tumors with chemotherapy a major challenge. As more effort is focused on understanding the cellular, molecular, and physiological mechanisms underlying drug resistance, it becomes clearer that a number of factors may be involved in the development of drug resistance. Studies at the cellular level have demonstrated that overexpression of membrane glycoproteins involved in drug influx/efflux (1–3), elevated levels of redox active molecules (4–6), and elevated activities of enzymes involved in detoxification (7, 8) can provide significant cellular resistance to chemotherapy drugs. The extent to which these biochemical adaptations contribute to drug resistance in vivo is at present not clear; however, they represent major targets for further study and possible clinical exploitation.

GSH and GSH-related enzymes are known to function in the cellular detoxification of potentially harmful xenobiotics and oxygen-related toxic species (9, 10). The importance of GSH in altering cellular response to certain chemotherapy drugs has been demonstrated by virtue of the development of agents that either inhibit (11) or stimulate (12) intracellular GSH synthesis. Depletion of intracellular GSH by BSO in a variety of cell types has been shown to markedly enhance the cytotoxicity of many chemotherapy drugs (13, 14) and hypoxic cell radiosensitizers (15). Conversely, elevating GSH levels prior to drug treatment by oxothiazolidine carboxylate can afford significant protection against chemotherapy drug-mediated cytotoxicity (4, 6, 16). Further evidence linking GSH with drug resistance has come from studies relating inherent intracellular GSH levels with drug sensitivity (17). Louie et al. (17) have shown that a human ovarian cell line made stably resistant to either melphalan, Adriamycin, or cisplatin by long term incubation in increasing concentrations of each respective drug had significantly higher GSH levels than the parent cell line. Drug resistance in each of these cell lines could be reversed by BSO-mediated GSH depletion (17). Human breast cancer cell lines resistant to Adriamycin exhibit increased GSH peroxidase and GSH transferase activity (7). Such findings have prompted the clinical exploration of GSH modulation of tumor cell GSH by BSO treatment. Phase I trials are currently under way evaluating BSO treatment in conjunction with melphalan for ovarian cancer.

The ongoing clinical trials centered around GSH modulation bring to attention several issues that will be important in interpreting the success or failure of such therapeutic strategies. (a) Do tumor cells in vivo actually have higher GSH levels than normal tissues? (b) Will BSO treatment result in sufficient GSH depletion to sensitize the tumor to the particular chemotherapy drug(s) used? (c) Will BSO treatment result in differential GSH depletion rates of tumor versus normal tissues? (d) Are there means of repleting normal tissue GSH levels after combination BSO/chemotherapy regimens? In order to answer these important questions accurate GSH measurements of tumor and normal tissues are imperative.

Accurate measurement of tumor GSH may be complicated by the presence of multiple cell populations within the tumor biopsy (18). Currently, the most frequently used methods for detection and quantitation of GSH include an enzymatic assay utilizing GSH reductase developed by Tietze (19) or high performance liquid chromatographic assays using fluorescent probes which react with GSH (20). Both assays are quite

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1 To whom requests for reprints should be addressed, at Radiation Oncology Branch/NCI, Bldg. 10, Room B3-B69, Bethesda, MD 20892.

2 The abbreviations used are: GSH, glutathione; BSO, buthionine sulfoximine; MCB, monochlorobimane; FCS, fetal calf serum; HBSS, Hanks’ balanced salt solution; PBS, phosphate-buffered saline; SSA, sulfosalicylic acid; GST, GSH S-transferase; SEM, standard error of the mean.
sensitive and may be appropriate for GSH measurements of homogeneous, in vitro cell lines; however, each assay measures only the average GSH content from a general population of cells and is less suited for specific analysis of the multiple cellular populations which may be found within a tumor. When two or more populations of cells are present in a sample the mean GSH value may over- or underestimate the GSH level of a particular population of interest. To determine GSH levels in subpopulations within tumor cell suspensions, measurements on a single cell basis with the use of MCB have been proposed (21). MCB is a nonfluorescent molecule which when conjugated to GSH (or other sulfhydryl containing compounds) becomes highly fluorescent. In rodent cell lines at low MCB concentrations (<100 μM) the reaction rate of GSH with MCB is greatly facilitated by the presence of a group of enzymes, glutathione S-transferases (21). These enzymes both accelerate the reaction and provide a high degree of specificity for GSH. Therefore, it should prove possible to use MCB to label GSH in tumor cell suspensions, determine the extent of GSH heterogeneity, and quantitate GSH levels in the various subpopulations of tumor cell suspensions.

We report here on the determination of tumor cell GSH levels from 27 surgically resected human lung tumors and surrounding normal lung tissue. Cell suspensions obtained by enzymatic digestion of these tissues revealed heterogeneous populations of cells with respect to cell type, cell volume, and GSH content. For most of the samples analyzed with the Tietze's assay, no significant differences in the average GSH content between tumor and normal lung cells were found with the exception of the squamous cell carcinomas. However, MCB labeling of cellular thiols coupled with flow cytometric measurements revealed subpopulations of cells within several of the squamous cell carcinomas which had significantly higher thiol levels than the surrounding normal lung tissue. These findings suggest that GSH/thiol levels may be quite variable in lung tumors and that an accurate assessment of tumor GSH levels may provide clinically important information with regard to tumor cell drug resistance.

MATERIALS AND METHODS

Cell Lines. A549 (human lung adenocarcinoma), HT-29 (human colon adenocarcinoma), and Morris hepatoma (rat hepatoma) cells were purchased from American Type Culture Collection, Rockville, MD. AG1522 (normal human fibroblasts) were purchased from the Human Genetic Mutation Cell Repository, Camden, NJ. SNU-C5 (human colorectal carcinoma) (22) cells were supplied by Dr. J. G. Park. MCF7-WT and MCF7-Adr² (human breast carcinoma) (7) were supplied by Dr. R. F. Ozols and the OVG1 (human ovarian carcinoma) cell line was initiated in our laboratory. B16(F1) cells (murine melanoma) were obtained from the Division of Cancer Treatment Tumor Repository, Frederick, MD. U-1 cells (human melanoma) were supplied by Dr. R. Weichselbaum. A4573 (human Ewing's sarcoma) cells (23) were supplied by Dr. T. Trishe. All of the above cell lines were grown in RPMI 1640 medium supplemented with 10% FCS, penicillin, and streptomycin. V79 (Chinese hamster lung fibroblasts) cells were grown in F-12 medium supplemented with 10% FCS, penicillin, and streptomycin. Exponentially growing stock cultures were maintained in a humidified atmosphere of 5% CO₂/95% air at 37°C. For studies involving GSH measurements, ~10⁶ cells were plated into 100-mm dishes and incubated for 24 h prior to collection.

Tissue Acquisition. All patients were referred to the Thoracic Oncology Section, Surgery Branch, National Cancer Institute, NIH, for resection of the primary or metastatic lung neoplasms. Portions of resected tumor and normal lung were removed for GSH measurements. A minority of the patients (see Table 2) had received some form of therapy, either radiation or chemotherapy, prior to resection.

Tumor/Normal Tissue Disaggregation. Tumor and normal tissues were surgically removed and processed within 1–3 h. Normal lung tissue was obtained distal to the tumor site when the surgical specimen was large enough to include both tumor and normal lung margins. Both tumor and normal tissue were minced into ~2-mm cubes with scissors and digested for 30 min with an enzyme cocktail consisting of 0.02% DNase I and 0.2% collagenase (Sigma Chemical Co., St. Louis, MO) in HBSS, pH 7.2, at 37°C with agitation. Following incubation undigested pieces of tissue were allowed to sediment and the supernatant cell suspension was collected and centrifuged at 800 x g to remove cells from the enzyme cocktail. Incompletely digested tissue pieces were resuspended in fresh enzyme cocktail and incubated for an additional 30 min as described above. Cell suspensions from both digestions were pooled and resuspended in 13 ml of 150 mM NaCl-10 mM KHCO₃-0.01 mM EDTA lysis buffer to remove the RBC. After 5 min the unlysed cells were centrifuged out of the above buffer, washed once with HBSS plus 5% FCS, and resuspended in HBSS plus 5% FCS for counting, volume measurements, and GSH assessment. The tissue disaggregation protocol consistently yielded single cell suspensions with viability > 80% as determined by trypan blue dye exclusion.

Flow Cytometry Analysis. Cells (10⁶ cells/ml) in PBS were obtained either by direct trypanosinization of in vitro cultures or in the case of tumor and normal tissue specimens by the tissue disaggregation technique described above. The cells were incubated with MCB (Molecular Probes, Inc., Junction City, OR) for 60 min at room temperature. After staining, the cells were washed once with PBS and then resuspended in 1 ml PBS for flow cytometry analysis. Samples were analyzed using a Coulter Epics V cell sorter (Coulter Electronics, Inc., Hialeah, FL) as described in a previous paper (24). For most specimens 10,000 to 20,000 cells were collected for analysis.

GSH Assay (Tietze). Single cell suspensions were obtained as described above and triplicate samples of 10⁶ cells were resuspended in 0.6% SSA for GSH analysis. Total GSH (reduced plus oxidized) was determined by the GSH cyclic reductase assay as described by Tietze (19). Protein was measured by the method of Bradford (25). GSH is expressed in nmol GSH per mg protein. The statistics for the Tietze's data are reported as the mean ± SEM.

Measurement of Protein Labeling with MCB. Cells (10⁶/ml) were labeled with 1 mM MCB for 1 h at room temperature. After one washing with PBS the cells were resuspended in either 1 ml PBS containing 0.2% Triton X-100 to measure the total fluorescence (protein plus nonprotein sulfhydryls) or resuspended in 0.6% SSA and placed on ice for 30 min to isolate total cellular protein. The SSA-treated cells were centrifuged and the pellet was resuspended in 1 ml PBS containing 0.2% Triton X-100 to measure the protein fluorescence.

The fluorescence for each sample was measured using a SLM 8000/34C spectrofluorometer (Urbana, IL). The bimane fluorescence was excited at 400 nm and the fluorescence was detected at 480 nm. Endogenous fluorescence from cells not treated with MCB but processed similarly to the MCB treated cells was analyzed and subtracted from the MCB treated cells.

Volume Analysis. Cell volumes of each cell line and of tumor/normal cell suspensions were determined by using an Elzone counter (Particle Data, Inc., Elmhurst, IL) calibrated with microspheres of known diameters.

RESULTS

Effect of Enzyme Digestion on GSH Levels. Because GSH was to be measured on a single cell basis enzymatic digestion of the tumor specimens was necessary. Tissue specimens often contained variable amounts of blood and since RBC contain GSH we thought it was necessary to remove (by lysis) the RBC to obtain more accurate tumor cell GSH values. In an effort to examine what effect the enzyme cocktail and RBC lysis buffer
had on GSH levels in cells, human lung adenocarcinoma cells (A549) were incubated for up to 1 h in the enzyme cocktail and resuspended in the RBC lysis buffer for 5 min to simulate the conditions of the tumor disaggregation and RBC lysis conditions. Incubating untreated cells in the RBC lysis buffer alone had minimal effects on the cellular GSH levels (93% of control). Incubation of cells for up to 1 h in the enzyme cocktail alone or in conjunction with the RBC lysis buffer (protocol followed for tumor samples) had essentially no effect on cellular GSH levels (99% of untreated control cells).

GSH Measurements of Tumor and Normal Lung Tissue. The GSH content as determined by the GSH cyclic reductase assay for 27 surgically resected human lung tumors and their adjacent normal lung tissue (24 samples were obtained) is shown in Table 1. To facilitate comparisons Table 1 is separated into 4 groups: group 1, adenocarcinomas; group 2, squamous cell carcinomas; group 3, large cell and small cell carcinomas and bronchial carcinoid; and group 4, lung metastasis from various tumors. The adenocarcinoma specimens (n = 8) had a GSH content of 8.86 ± 0.96 nmol/mg protein while the matching normal lung specimens had a GSH content of 10.64 ± 0.94 nmol/mg protein. The squamous cell carcinoma specimens (n = 8) had a GSH content of 23.25 ± 5.99 nmol/mg protein compared to their normal lung specimens with GSH levels of 13.13 ± 1.23 nmol/mg protein. The small cell carcinoma, large cell carcinoma, and bronchial carcinoid specimens (n = 6) had a GSH content of 12.34 ± 2.40 nmol/mg protein compared to mean GSH levels of 9.65 ± 1.11 nmol/mg protein for their normal lung specimens (n = 5). Of the specimens analyzed, 4 patients' tumors were found to have GSH levels greater than 2-fold that of their corresponding normal tissue. These included 2 squamous cell carcinomas (No. 1557 with 23.4 nmol GSH/mg protein and No. 1534 with 62.9 nmol GSH/mg protein), a bronchial lung carcinoind (No. 1287 with 21.4 nmol GSH/mg protein), and a metastatic osteogenic sarcoma to the lung (No. 1196 with 33.9 nmol GSH/mg protein). For comparison, exponentially growing Chinese hamster V79 cells had a GSH content of 29–35 nmol GSH/mg protein while an established human ovarian carcinoma line (OVG-1) in exponential growth had a GSH content of 175–234 nmol GSH/mg protein. Differences in GSH levels between tumor biopsies and in vitro tumor cell lines have been reported previously (18).

Variability of Cell Types from Tumor/Normal Lung Digests. Because Tietze's assay provides only an average measurement of GSH content within a population of cells, the presence of multiple populations each having a unique GSH content could not be easily identified and measured. That multiple populations of cells were present in both the tumor and normal lung tissue can readily be seen by representative volume analysis of specimens (Fig. 1). Cells isolated from normal lung consistently showed the presence of at least 3 populations while the tumor specimens were generally much more variable (2–3 peaks). We have tentatively identified the peak at channel 29 as lymphocytes since WBC isolated from human peripheral blood shows a peak at channel 29.

Use of MCB to Measure Cellular GSH Levels. Recently the dye, MCB, has been introduced as a means of specifically labeling GSH in cells with a fluorescent probe. When MCB is used in conjunction with flow cytometry it becomes possible to examine the GSH heterogeneity profile of a cellular population (21). Previous work has indicated that when the GSH levels in rodent cell lines were depleted or elevated by known amounts and analyzed by either the GSH cyclic reductase assay or the MCB assay that an excellent quantitative correlation existed between the two assays (21, 25). These data suggested that by

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**Table 1 Human tumor and normal lung tissue GSH data**

<table>
<thead>
<tr>
<th>No.</th>
<th>Patient</th>
<th>Tumor type</th>
<th>Treatment status</th>
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<tr>
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<td>Adenocarcinoma</td>
<td>No</td>
<td>12.28 12.28</td>
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<td>No</td>
<td>10.44 13.74</td>
</tr>
<tr>
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</tr>
<tr>
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</tr>
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<td>1427</td>
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<td>Cyt/Adria</td>
<td>9.36 12.57</td>
</tr>
<tr>
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<td>1492</td>
<td>Primary pulmonary sarcoma</td>
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<td>27.36 14.33</td>
</tr>
</tbody>
</table>

* VP, etoposide; CP, cis-platinum; Cyt, Cytoxan; RT, radiation therapy; Adria, Adriamycin.

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Fig. 1. Volume analysis of cell suspensions from normal lung and tumor specimens. A, normal lung cells; B, lung metastases from a soft tissue sarcoma; C, lung metastases from a osteogenic sarcoma. Volume analysis of human peripheral WBC shows a peak at channel 29.
cell types examined in the protein fluorescence never exceeded GSH concentration was much reduced. Because bimane fluorescence measures the total thiol content of cells and the GSH cyclic reductase assay values corrected for protein content (nmol GSH/mg protein) and the ratio of total GSH content ($R^2 = 0.84$) (Fig. 2). Below 80 nmol GSH/mg protein cellular bimane fluorescence increased linearly with increasing GSH content ($R^2 = 0.82$). However, above 80 nmol GSH/mg the rate of change in the cellular fluorescence with respect to increasing GSH concentration was much reduced.

For MCB concentrations greater than 100 $\mu M$ the nonenzymatic reaction to MCB with both nonprotein sulfhydrils (GSH) and protein sulfhydrils is important (26). To examine the extent of protein sulfhydril reactions with 1 mM MCB, A549 cells, MCF7WT cells, or human lymphocytes were labeled for 1 h at room temperature and the percentage of the total bimane fluorescence due to protein labeling was determined. Of the 3 cell types examined the protein fluorescence never exceeded 25% of the total fluorescence (Table 2). Overall, when human cells are used the MCB technique cannot be used to quantitatively measure the GSH content; however, qualitative GSH/thiol results still appear to be a realistic possibility in cells with a nonperturbed GSH pool.

**MCB Labeling of Tumor/Normal Lung Specimens.** Because the MCB technique offered the possibility of qualitatively analyzing GSH/thiol heterogeneity within the tumor biopsies, both tumor and normal tissue specimens were stained with MCB and analyzed by flow cytometry. Fig. 3 shows MCB labeling of 4 different tumor biopsies: 2 adenocarcinomas (Nos. 1163 and 1531) and 2 squamous cell carcinomas (Nos. 1534 and 1557) of the lung, along with the normal lung cells associated with each specimen. Since the normal tissue and respective tumor samples were collected, processed, stained, and analyzed on the same day direct comparison between the fluorescence profiles of the tumor specimens to the normal lung cells was done; however, comparison between different lung tumor specimens is not possible because samples were done on different days. Even with the inherent problems associated with the analysis of a large number of samples collected over a long period of time, several observations can be made concerning these profiles. (a) In many if not most of the tumor specimens studied, several fluorescence peaks were noted which indicate the pos-

![Graph](image-url)
sibility of quantitative differences in thiol content among the different subpopulations of cells within the tumor biopsy (Fig. 3, A, E, and G). Multiple fluorescence peaks were also noted in the normal lung specimens and the relative proportion of cells in these peaks varied from patient to patient (Fig. 3, B, D, F, and H). (b) In a majority of specimens examined, the mean bimane fluorescence values calculated for tumor cells were less than the calculated mean of the corresponding normal lung cells. However, in 2 of the squamous cell carcinoma samples studied, a distinct population of cells was identified as having a significantly higher fluorescence than any cells in the corresponding normal lung cell profiles (Fig. 3, E, G).

Because the MCB fluorescence profiles are a measure of total thiol content and not thiol concentration it is not possible to state with certainty that the peaks seen in Fig. 3 represent populations with different thiol concentrations. However, if the volume and thiol content of each subpopulation of cells were known then a more definitive statement regarding the heterogeneity of thiol concentrations between cell populations could be made. Therefore, in an effort to correct the fluorescence for size differences, the fluorescences from cells with identical or near identical light scatter properties were collected and analyzed (Fig. 4). Although not linearly related to size, the light scatter signal collected from cells did provide a normalization technique for size measurement which can be used for comparison between different cell populations (27). The first squamous cell sample (No. 1534) shown in Fig. 4 demonstrated that for cells of similar sizes (cross-hatched area) a population of cells (35%) with >5 times the fluorescence intensity of normal lung cells was identified. A second squamous cell carcinoma sample (No. 1557) was analyzed and found to be very similar to the data shown in Fig. 4 (data not shown); however, unlike the first sample, the proportion of cells having high fluorescence values was much lower (10%) (Fig. 3G).

As shown in Fig. 3, the tumor and normal lung specimen MCB profiles showed significant variation from patient to patient. To study whether these differences were due to changes in the composition of normal lung cell populations or to a MCB staining artifact, both volume distributions (as measured with a particle data counter) and forward angle light scatter information were collected for tumor and normal cell populations. The relative proportions of cells were seen to shift from patient to patient when analyzing either light scatter or volume measurements and did not appear to be due to any staining artifact (data not shown).

DISCUSSION

Previous studies have shown that GSH can and does function to reduce the cellular toxicity of various chemotherapy agents (13, 17). On the basis of this evidence investigators have postulated that one form of drug resistance may result from elevated levels of GSH in tumor cells (28, 29). Whether high tumor GSH levels contribute to drug resistance upon initial treatment or at relapse is not known. Before such important issues may be addressed, techniques must be developed to accurately measure tumor GSH levels. In this study we have analyzed the GSH content of a number of lung tumors and normal lung tissue. With the possible exception of some squamous carcinomas, in general, tumor GSH levels were found to be not significantly higher than those for normal lung. The presence of subpopulations of cells having high GSH levels within tumor samples has been documented by the current work. However, several concerns and limitations regarding the measurement of GSH from tumor biopsies and how these data may be interpreted are raised by our findings.

Inherent in the process of tissue disaggregation to single cell suspensions is the potential for loss, degradation, or oxidation of the molecule of interest. There are a number of techniques available for tissue disaggregation involving use of different enzymes and variable lengths of exposure (29, 31). To avoid major changes in GSH concentrations during the cell isolation period, a high activity enzyme cocktail was applied for a short time. The GSH levels in A549 cells incubated for 1 h in the enzyme digest buffer was reduced by less than 10%. Variation in the number of RBC [GSH concentrations between 1 and 3 mm (32)] posed another potential problem. Although the total amount of GSH is small for each individual RBC, large and varying numbers of RBC in tissue samples could have an impact on the interpretation of GSH values for the cells of interest. The simplest means of addressing the RBC problem was to selectively remove the RBC from the samples by lysis. Treatment of A549 cells with the enzyme cocktail in conjunction with the RBC lysis buffer also had minimal effects on the cellular GSH levels. In addition to the in vitro data, the GSH cyclic reductase results from the tumor and normal tissue biopsies suggest that the perturbations was not severe because: (a) the normal lung GSH values were very uniform from patient to patient; and (b) in the majority of the lung cases studied there was no significant GSH differences between the tumor and normal lung tissue. Furthermore, the GSH levels for both the tumor and normal tissues were in a range previously reported both for lung tumors and other tumor types (18, 33–34). We believe that while the absolute GSH values listed in Table 1 may have some error associated with them, the relative GSH differences between tumor and normal lung specimens should be reasonably accurate.

Another important variable in tumor GSH measurements was the presence of multiple populations of cells found in both tumor and normal lung specimens. This was immediately apparent from the analysis of cell suspension volume data (Fig. 1) and from initial studies using an anti-panleukocyte monoclo-
nal antibody to establish that leukocytes (B-cells, T-cells, macrophages, and neutrophils) are present in tumor samples. Because leukocytes have low GSH levels (18), their presence in tumor specimens would be expected to lower the average GSH measurements determined by the Tietze's assay. The composition of other cell populations is unknown. Histological examination of each tumor specimen indicated that tumor cells were present; however, there may be different subpopulations of tumor cells present as a result of environmental and nutritional constraints within the tumor as well as populations of proliferating and nonproliferating cells. Additionally, normal host fibroblasts could be present. All of these variables can have an impact on the GSH status of the population of cells measured (18, 33).

Another question associated with human tumor GSH measurements is what cell types, if any, the tumor cells should be compared against. From a therapeutic view, tumor GSH levels should be compared with that of normal tissue(s) with the lowest levels and therefore presumably at the greatest risk for toxicity. However, obtaining samples of normal tissue is not always feasible. Although it was not entirely ideal, we were fortunate to have for comparison normal lung tissue obtained from a location distal to the tumor site for comparison. GSH levels measured in normal lung cells from 24 patients were very similar even though the size information indicated that the relative proportion of each population of cells found in normal lung samples varied from patient to patient. In addition, the fluorescence profiles for the normal lung samples also showed significant differences from patient to patient (Fig. 3). These conflicting results could be explained if the cellular populations of the normal lung had similar GSH concentrations.

For many of the tumor specimens analyzed, little or no difference was found between the GSH content of the tumor and the surrounding normal lung cells (Table 1). However, the possibility remains that a limited subpopulation of tumor cells with high GSH levels could be present in tissue but, by virtue of their limited numbers, would go undetected by the averaging nature of the GSH cyclic reductase analysis. Multiple peaks in the fluorescence profiles were indeed seen and the possibility that cells with differing thiol levels in both tumor and normal specimens may be a frequent occurrence is supported by such multiplicity (Fig. 3). In a majority of the samples studied, the tumor specimens did not have populations of cells with a bimane fluorescence exceeding the bimane fluorescence of cells composing the normal populations (Fig. 3, A–D). Thus, for these samples, it would not appear that tumor cell subpopulations with higher than normal thiol levels were present. Even though no significant differences in GSH levels were seen in many of the tumor specimens when compared to normal lung it is still possible that patients developing resistance to chemotherapy agents may show an increasing subpopulation of cells with elevated levels of GSH as treatment time progresses. Another possibility is that the tumor does not have constitutively higher levels of GSH but is equipped to rapidly and more readily synthesize GSH if and when stressed by chemotherapeutic drugs.

MCB has been shown previously to label the GSH pool in rodent cell lines and to be an excellent indicator of the GSH status when GSH is modulated by either BSO (to deplete) or GSH esters (to elevate) (21, 24). However, our experience has been that the concentrations used to label rodent cell lines are entirely inadequate for GSH labeling in human cell lines (24, 26). In fact, we have found that concentrations up to 1 mM MCB are required for maximal GSH labeling in human cells, a concentration some 20–100 times greater than that used for the labeling of rodent lines (24, 26). When 1 mM MCB was used to label various human and rodent cell lines the fluorescence intensities were correlated to GSH levels in cell lines with 80 nmol GSH/mg protein or less (Fig. 2). Above 80 nmol GSH/mg protein changes in the fluorescence intensities with respect to increasing concentrations of GSH were reduced (Fig. 2). Since 1 mM MCB labels from 60 to 90% of the GSH pools in these human tumor cell lines only a small fraction of the differences between the GSH cyclic reductase results and fluorescence results can be explained by inadequate GSH labeling (26). Because of the differences between the fluorescence and the GSH cyclic reductase results, we stress that using the published conditions for labeling GSH with MCB (21) and those we have optimized for the present study (24, 26), the technique cannot be used as a quantitative assay for measuring the GSH levels in human cells.

Although not usable as a quantitative GSH assay the MCB assay can provide some useful qualitative cellular thiol information. Therefore, comparisons were made between normal lung cells and tumor cells which were processed, stained, and analyzed on the same day. In 2 squamous cell lung carcinoma samples (Nos. 1534 and 1557) the bimane fluorescence profiles showed the presence of a subpopulation of cells having a much higher fluorescence intensity (>5 times) than cells from the normal lung (Fig. 3, E, G). The elevated fluorescence intensities could have resulted from several possibilities. First, cells with equal thiol concentrations but different volumes would have different fluorescence profiles due to the fact that the MCB-flow cytometry assay measures the thiol content of cells. However, when cells of similar sizes from both normal and tumor populations were analyzed (Fig. 4, B, D) the subpopulation of tumors cells with elevated fluorescence intensities was still present, thus supporting the proposal that within this tumor there was a subset of cells with truly different thiol concentrations.

Second, because the MCB/GSH reaction is catalyzed by the GSH S-transferases (21, 26, 35), enzyme differences (either quantitative or catalytic) could have resulted in the elevated fluorescence intensities. This possibility was considered unlikely inasmuch as the labeling times were long (1 h) and the concentration of MCB used was high. In addition, we have shown in MCF7WT cells, which contain very low (or undetectable) GST activity, that the percentage of GSH labeled by MCB was similar to that in the MCF7ADR cells which contain very high GST activity (7, 26). Thus, at the higher MCB concentrations, MCB labeling of GSH occurs predominantly by nonenzymatic means and therefore should not be heavily influenced by the presence or absence of GST activity (26).

Third, because of the high MCB concentrations needed to label GSH in human cells it is possible that cellular protein thiols and other nonprotein thiols (such as cysteine) may contribute substantially to the bimane fluorescence profiles. We do not believe that bimane protein fluorescence represents a significant portion of the tumor or normal lung flow fluorescence profiles since results from Table 2 suggest that at most only 20–30% of the MCB-derived fluorescence is due to protein labeling. Furthermore, by high performance liquid chromatographic analysis the fluorescence profile of several MCB-stained tumor specimens revealed that the bimane-GSH adduct was the predominant bimane adduct present (>85–95% of the total
nonprotein sulfhydryls labeled. Most importantly, in patient 1534 the GSH content of the tumor cells measured by the GSH cyclic reductase assay was much higher than the normal lung cells (62.9 versus 8.8 nmol/mg protein). This suggests that the population of cells with increased fluorescence intensities seen in Fig. 3, E, G, was due to real GSH differences. Therefore, while additional work must be done to conclusively demonstrate elevated GSH levels within tumor specimens, our data provide evidence for and is consistent with the proposal that tumors can have a subpopulation of cells with elevated GSH levels.

Since only 35% of the cells had elevated fluorescence intensities it is likely that much larger GSH differences actually existed in the tumor cells (Fig. 3E). The proposal that the presence of a subpopulation of cells with high GSH levels within a tumor could be missed by any averaging technique is illustrated by the second squamous cell case (patient 1557) in which the actual measured difference in GSH levels between the tumor and normal tissue was much less (25.3 versus 16.3 nmol GSH/mg protein). For this tumor only 10% of the cells had elevated fluorescence intensities in the flow cytometry profile (Fig. 3G). These data would suggest that when utilizing averaging techniques to measure GSH it is possible to overlook significant GSH differences between tumor and normal cells.

Finally, associated with GSH measurements of tumor specimens is intratumor GSH heterogeneity. A recent report by Lee et al. (18) has emphasized the fact that GSH levels can vary depending upon the location of the biopsy. Using human ovarian xenografts, differences in GSH levels from 4- to 7-fold were observed when individual pieces of the same tumor were compared (18). They suggested that such differences might in part result from the growth status among cells in different regions of the tumor and showed that plateau phase cells have much lower GSH levels than exponentially growing cells (18). GSH variability may also be present in the data presented in this report; however, without exception the tumor GSH values measured were either very similar to the normal lung tissue GSH values or higher.

In conclusion, we have measured the GSH content of both tumor and normal tissue specimens using a combination of Tietze's and MCB analyses. In many of the tumor specimens analyzed the GSH levels as measured by the Tietze's technique were not different from normal lung tissue. The MCB technique indicated the possible presence of cells having differing thiol levels. Most of the tumor specimens analyzed did not have cells with thiol levels exceeding cells in the corresponding normal tissue. The MCB and GSH cyclic reductase data in 2 squamous cell carcinoma cases indicated that cells with elevated GSH levels were present in the specimens. While the MCB technique has several problems when used to stain human cells, it may offer the best hope in identifying GSH heterogeneity in the multiple populations found in human tumor and normal specimens.

REFERENCES


Cellular Glutathione and Thiol Measurements from Surgically Resected Human Lung Tumor and Normal Lung Tissue

John A. Cook, Harvey I. Pass, Susan N. Iype, et al.


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