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Glutathione Metabolism in Patients with Non-Small Cell Lung Cancers

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ABSTRACT

Non-small cell lung cancer (NSCLC) is the leading cause of cancer death in the United States. Because NSCLC is highly chemoresistant, it is usually not treatable. Altered glutathione (GSH) metabolism is thought to be one major mechanism of chemoresistance, and GSH levels are reported to be elevated in NSCLC. The main objective of this study is to delineate the potential mechanisms involved in elevation of tissue GSH, including extraction from the circulation by NSCLC. Twenty consecutive patients with NSCLC were enrolled. At the time of lobectomy, pulmonary artery and vein were identified, and blood flow was measured by an electromagnetic probe. Subsequently, blood samples were drawn from pulmonary artery, the vein draining the tumor-bearing lobe, and a normal lobe. Immediately after lobectomy, tumor and lung specimens were snap frozen. NSCLC tumor specimens had higher levels of GSH compared with lung tissue (20.8 ± 9.4 *versus* 11.6 ± 3.0 nmol/mg protein, respectively; $P < 0.05$). The tumor demonstrated higher activity of the enzyme γ-glutamyl transpeptidase, a membrane-bound enzyme involved in transmembrane uptake of GSH, than lung tissue (41.9 ± 26.4 *versus* 22.4 ± 12.3 units/mg protein, respectivel; $P < 0.05$). Also, the tumor-bearing lobe showed elevated extraction of GSH and two of its component amino acids compared with lung tissue (GSH uptake: 0.60 ± 0.67 *versus* 0.20 ± 0.40 μM/min, respectively; $P < 0.05$). NSCLC tumors are able to extract circulating GSH and its constituent amino acids to synthesize intracellular GSH. Increased activity of γ-glutamyl transpeptidase may be one mechanism underlying increased GSH uptake by NSCLC.

INTRODUCTION

Lung cancer is the leading cause of cancer-related deaths and causes more than 125,000 deaths annually in the United States alone. Because most patients are diagnosed in advanced stages of the disease, the overall 5-year survival is only 15%. NSCLC[2] comprises 80% of the primary lung cancers. At present, complete surgical resection remains the only hope for cure, with chemotherapy or radiotherapy having minimal impact on survival.

GSH metabolism has been postulated to contribute to resistance in radiation and chemotherapy in a wide variety of neoplastic tissues (1, 2). GSH is a tripeptide formed from the amino acids glutamine, cysteine, and glycine, and is the most prevalent intracellular free radical scavenger. GGTP is a membrane-bound enzyme that hydrolyzes the γ-glutamyl bond of glutathione and transfers the γ-glutamyl amino acid into the cell where it is resynthesized into GSH (3, 4). GSH and its related enzymes have been shown to be elevated in several human cell lines, including NSCLC cells (5–7). Furthermore, our laboratory has demonstrated that mammalian tumors are able to use increased amounts of circulating GSH and that this heightened cellular extraction may be mediated through the increased activity of the enzyme GGTP (8). We designed a unique *in vivo* human model to achieve two main objectives: (*a*) to measure extraction of GSH and its amino acid constituents across *in situ* primary lung cancer and surrounding normal lung; and (*b*) to investigate the possible mechanisms involved in elevation of tissue GSH levels in NSCLC.

MATERIALS AND METHODS

Clinical Material. Twenty consecutive patients with presumptive or biopsy-proven NSCLC, scheduled to undergo curative lobectomy, were entered in this study after signing of informed consent. There were 12 woman and 8 men with an average age of 62.5 ± 10.9 years. Eighteen patients had a smoking history, and two patients had preoperative chemotherapy. The procedures followed in this study were in accordance with the ethical standards of the Committee on Human Experimentation at Memorial Sloan-Kettering Cancer Center and were based on an Institutional Review Board protocol approved by the Department of Health and Human Services.

Experimental Design. Surgical dissection for the planned lobectomy was performed in a standard fashion. The pulmonary artery and vein pertaining to the tumor-bearing lobe and normal (tumor-free) lobe were identified and isolated. An electromagnetic flow probe [models SF430, SF435, or SF445, Carolina Medical Instruments, Inc., King, NC; Ref. 9] was placed around the pulmonary artery and/or the draining pulmonary vein from the corresponding lobe. In the first five patients, we studied flow through both the artery and vein and found no difference. Subsequently, flow was measured only from the pulmonary vein. If an adequate probe fit could not be attained, the patient was excluded from the study. Blood samples were drawn in heparinized syringes from the main pulmonary artery, a branch of the pulmonary vein draining the tumor-bearing lobe (TPV) and a branch of the vein draining a tumor-free lobe (NPV). Blood samples were immediately centrifuged (Fisher Scientific, Pittsburgh, PA) at 9000 rpm for 2 min. A portion of the supernatant was immediately deproteinized with cold 10% trichloroacetic acid in preparation for GSH analysis. The remainder of the supernatant was aliquoted for subsequent glucose, lactate, and amino acid measurements and stored at −80°C. Lobectomy was completed, and the resected lobe was transferred to a side table where the surgeon carefully excised a sample of tumor tissue and adjacent normal lung from the lobectomy specimen. To minimize contamination of tumor from lung, a sample of tumor tissue was removed from the center of the *in situ* tumor. The specimens were then snap frozen in liquid nitrogen and stored at −80°C.

Tissue extraction was defined as the pulmonary arterial concentration − pulmonary venous concentration multiplied by the blood flow:

$$\text{Extraction} = (\text{Conc}_{\text{art}} - \text{Conc}_{\text{vein}})Q,$$

where Q was measured in liters/min.

GSH Measurement. Reduced GSH was measured by the method described previously by Hissan and Hilf (10). Briefly, 50 mg of each tissue specimen were homogenized on ice for 30 s (Polytron, Kinotica, Luzern-Schweiz, Switzerland) in 1 ml of 5% trichloroacetic acid. Previously deproteinized plasma samples and the freshly prepared tissue homogenates were centrifuged at 3000 × *g* for 15 min. Two hundred μl of each supernatant were mixed with 1.7 ml of phosphate buffer [sodium phosphate 0.1 M/EDTA 5 mM (pH 8.0)] and 100 μl of *O*-phthaladehyde (1 mg/ml in absolute methanol). After a 15-min dark incubation, the sample fluorescence was read at 350 nm excitation and 420 nm emission. The concentration of GSH was determined using a known standard curve. All measurements were performed in duplicate.

γ-Glutamyl Transpeptidase Analysis. Tumor and normal lung tissue were homogenized (Polytron) in PBS, and the homogenates were used to determine GGTP activity by means of spectrophotometric assay. The reaction was performed at 37°C for 20 min and used L-γ-glutamyl-*p*-nitroanilide (Sigma, St. Louis, MO) as the substrate and glycylglycine as the acceptor (11). One unit of GGTP is defined as that amount of activity that liberates 1 nmol

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[2] The abbreviations used are: NSCLC, non-small cell lung cancer; GSH, glutathione; GGTP, γ-glutamyl transpeptidase; TPV, tumor pulmonary vein; NPV, normal pulmonary vein; GST, glutathione *S*-transferase.

Table 1 *Patient and tumor characteristics*

Patient no.	Sex	Type[a]	Stage	GSH tumor nmol/mg protein	GSH lung nmol/mg protein	GGTP tumor units/mg protein	GGTP lung units/mg protein	Extraction tumor μM/min	Extraction lung μM/min
1	F	Adeno	T1N0M0	14.4	13.2	17.7	12.2	0	0
2	M	Squam	T2N0M0	13.5	11.2	75	15.7	0.43	0
3	F	Bronchal	T2N1M0	20.3	11.1	91.6	36.2	0.57	0.47
4	F	Neuroend	T3N2M0	30.5	13.1	59.8	18.3	1.93	0
5	M	Bronchal	T3N0M0	19.6	13	33.1	48.2	0.40	0.52
6	F	Adeno	T2N1M0	14.2	10.3	24.1	18.4	0	0
7	M	Squam	T3N0M0	34.4	17.1	119	10.8	2.1	0
8	F	Adeno	T2N2M0	15.3	12.0	45.6	30.1	0	0
9	F	Squam	T3N0M0	28.4	8.3	59.3	16.9	1.2	0.37
10	M	Squam	T1N0M0	17.1	18.0	15.8	14.5	0	0
11	M	Adeno	T2N0M0	12.8	9.7	42.3	5.3	0	0
12	F	Adeno	T1N0M0	27.8	8.2	64.6	3.15	1.54	0
13	F	Large	T2N0M0	15.0	13.0	29.6	31	0.68	0.98
14	M	Sqam	T1N1M0	14.6	16.7	20.5	23.9	0	0
15	M	Adeno	T2N2M0	41.4	8.4	39.4	24.6	1.15	0
16	F	Adeno	T3N2M0	15.0	8.5	40.6	44.0	0.49	0.19
17	M	Adeno	T2N0M0	26.6	8.6	38.7	20	0.69	0
18	F	Neuroend	T2N2M0	5.4	8.7	37.6	45.5	0.90	1.45
19	F	Squam	T2N0M0	35.6	6.5	28.0	23.6	0.05	0.03
20	F	Adeno	T2N0M0	23.4	13.0	31.3	27.7	0	0

[a] Adeno, adenocarcinoma; Sqam, squamous cell carcinoma; Bronchal, bronchalveolar carcinoma; Large, large cell carcinoma.

of *p*-nitroaniline per minute, measured at 540 nm. An aliquot of the homogenate was used to determine protein concentration by a modified Pierce method (12).

Analysis of Amino Acids, Glucose, and Lactate. Plasma-free amino acid concentration was determined by high-pressure liquid chromatography using Pico.tag (Waters, Milford, MA) method as described previously (13). Briefly, plasma or known standard solution was mixed in a 1:1 ratio with internal standard solution (methionine sulfone, 0.4 mM in 0.1 M HCl) followed by filtration through Nanospin plus centrifugal filters (Gelman Science, Ann Arbor, MI). Twenty-five μl of filtered sample or standard was pipetted into a 6 × 50-mm sample tube and dried in the vacuum station. Fifteen μl of drying solution (methanol: 1 M sodium acetate: triethylamine in a 2:2:1 ratio by volume) was added to each tube and dried under vacuum. Twenty μl of the derivatization reagent (methanol: 1 M sodium acetate:triethylamine:water:phenylisothiocyanate in a ratio of 7:1:1:1 by volume) was added to each dry sample and incubated for 20 min at room temperature. After complete drying, the sample was diluted with 100 μl of Pico.tag diluent [5 mM Na_2HPO_4 (pH 7.4)] mixed with acetonitrile (ratio of 95:5 by volume). The solution was then injected into high-pressure liquid chromatography apparatus. The system consisted of Waters pump 510, Autosampler 717, and Temperature Control Module. Detection was performed with Waters 486 tunable absordetector at 254 nm (Waters). Data were collected and calculated by Millennium version 2.10 program (Waters). Ten μl of sample were injected onto the column (Pico.tag amino acid analysis column, 3.9 × 300 mm, Waters) in a column heater insert using a dual solvent system and a flow rate of 1 ml/min. Eluent A consisted of 0.13 M sodium acetate trihydrate and acetonitrile in a ratio of 47:3. Eluent B consisted of acetonitrile and Milli-Q quality water in a ratio of 60:40. The gradient was as described previously. Retention times for peaks of interest were the following: glycine 10.2 min, glutamine 11.2 min, and cysteine 52.3 min (13). Glucose and lactate were measured as described previously (14, 15).

Statistics. All data are presented as the mean ±SD. Comparisons between tumor and normal lung were performed using Mann-Whitney U test. A $P < 0.05$ was regarded as significant. Linear regression was used to assess correlation between tumor size and glucose extraction.

RESULTS

Tumor Characteristics. Pathological stage and histology are shown in Table 1. The mean tumor size was 3.77 ± 1.9 cm, corresponding with an average volume of 155 ± 220 cm^3. Blood flow was equivalent between the tumor-bearing lobe (0.75 ± 0.3 liter/min) and the normal lobe (0.74 ± 0.4 liter/min).

Substrate Flux. Tumor extracted higher amounts of glucose than normal lung; however, the difference was not significant (Table 2). Furthermore, the amount of glucose extracted by the tumor-bearing lobe correlated linearly with the tumor volume ($R^2 = 0.88$, $P < 0.001$; Fig. 1). Tumor released more lactate (−4.7 ± 7.1 mg/min) than lung (0.65 ± 9.0 mg/min), and this difference approached significance ($P = 0.069$; Table 2).

Amino Acid Flux. Tumor extracted significantly higher amounts of glutamine and glycine than normal lung. There was no significant difference in cysteine uptake between tumor and normal lung (Table 2).

Tissue GSH and GGTP Levels. Tumor tissue contained significantly higher levels of GSH (20.8 ± 9.4 nmol/mg protein) compared with lung tissue (11.6 ± 3.0 nmol/mg protein, $P < 0.05$; Table 1). The tumor, also, had significantly higher activity of the enzyme GGTP (41.9 ± 26.4 *versus* 22.4 ± 12.3 units/mg protein, $P < 0.05$; Table 1). There was no correlation between tumor size and tissue level of GSH.

GSH Extraction. The tumor-bearing lobe demonstrated higher extraction of GSH than the tumor-free lobe (0.60 ± 0.67 μM/min *versus* 0.20 ± 0.40 μM/min, $P < 0.05$; Table 1).

DISCUSSION

Other investigators have demonstrated high extraction of several energy substrates in various types of human tumors. Norton *et al.* (16)

Table 2 *Substrate flux across NSCLC*

Blood flow was measured through the TPV and NPV. Blood samples were taken from the branch of the pulmonary artery, TPV, and NPV. Extraction was defined as $(Conc_{art} - Conc_{vein})Q$, where Q was measured in liters/min.

	Glucose (mg/min)	Lactate (mg/min)	Glutamine (mmol/min)	Glycine (mmol/min)	Cysteine (mmol/min)
Tumor	53.9 ± 29.8	−4.7 ± 7.1	0.051 ± 0.054[a]	0.025 ± 0.038[a]	0.02 ± 0.10
Lung	31.8 ± 58.4	0.65 ± 9.0	0.013 ± 0.041	0.002 ± 0.01	−0.01 ± 0.09

[a] $P < 0.05$ tumor *versus* lung.

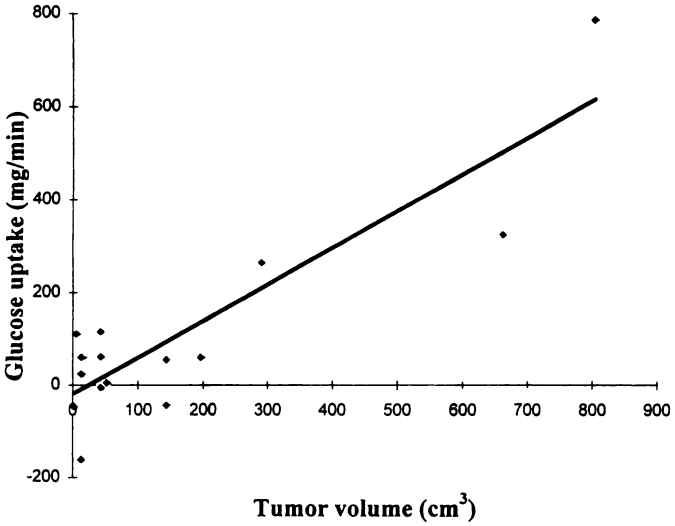

Fig. 1. Correlation of glucose uptake and tumor volume. There is a positive correlation between glucose uptake and tumor volume. The greater the tumor volume the higher the glucose uptake ($R^2 = 0.88$, $P < 0.001$).

showed high glucose extraction across sarcoma-bearing limbs in humans. Similarly, Holm *et al.* (17) demonstrated that *in situ* colon carcinomas were highly active in glucose metabolism and released high levels of lactate into the circulation (18). These studies reproducibly illustrated the heightened metabolic state of tumor tissue and introduced several novel methodologies allowing measurement of *in vivo* substrate utilization across tumor bed. Our model provides an opportunity for direct measurement of blood flow to tumor-bearing and normal tissue and creates a unique setting to investigate substrate flux across tumor bed. Because each lobe of the lung has a separate blood supply, we were able to directly compare the metabolic activity of a tumor-bearing lobe to a tumor-free lobe.

To study the metabolic activity of the primary lung cancer, we studied the flux of two energy substrates, *i.e.*, glucose and lactate, across the tumor bed. We showed a positive correlation between tumor size and glucose uptake. We did not observe a significant difference in glucose uptake or lactate release between tumor-bearing and normal lung; however, there was a trend that the tumor-bearing lobe extracted more glucose and released more lactate. One possible explanation for these findings is the relatively small size of the tumors studied in this experiment. Larger differences could be expected in bulkier tumors, as has been reported previously for other types of tumors.

There is a paucity of information about the *in vivo* metabolism of GSH in human lung and primary lung cancer. Animal data suggest that normal lung is capable of effectively using the circulating GSH, and the majority of this extraction is mediated by transmembrane breakdown of GSH by the enzyme GGTP. Subsequently, GSH is intracellularly resynthesized (19). Elevated levels of GSH and GSH detoxifying enzymes, such as GST and glutathione peroxidase, have been demonstrated in NSCLC and have been hypothesized to contribute to tumor chemoresistance (6, 7, 20). Britten *et al.* (21) found a 10-fold increase in GSH levels in human ovarian tumor cells obtained after alkylating agent resistance had developed. Previous research has revealed that patients with NSCLC who had undetectable or low serum GST levels responded to chemotherapy significantly better than those with high serum GST levels (20). Similarly, Bai *et al.* (22) demonstrated that in NSCLC, patients with negative GST tumors responded significantly better than those with positive GST tumors. Tumors may increase their detoxifying ability and intracellular stores by extracting GSH from serum. GGTP is an enzyme essential for transport of extracellular GSH into the cell (3, 4). High activity of this enzyme has been reported previously in other types of cancers (23, 24). *In vitro* research shows that up-regulation of GGTP activity in tumor cells may be related to activation of the *ras* proto-oncogene. Transfection of cells with *ras* results in the induction of GGTP in rat liver tissue (25). The delineation of the relationship of GGTP activity and *ras* activation in NSCLC needs further study.

The present study is the first to examine these parameters *in vivo*. As reported previously, our experiments show significantly higher tissue GSH levels in NSCLC compared with normal lung (5, 6); however, this is the first report showing elevated GGTP enzyme activity in NSCLC. These results may underestimate the differences between tumor and lung. We were careful to sample tumor from the core of the *in situ* specimen, but a few lung cells may have contaminated our tumor specimens and diluted our results. Furthermore, we demonstrated that tumor-bearing lung is capable of extracting higher amounts of GSH from the pulmonary circulation compared with normal lung. These findings are substantiated by the observation of elevated GGTP enzyme activity in the primary lung cancer, potentially a mechanism for intracellular trafficking of circulating GSH. D,L-Buthionine-*S*, *R* sulphoximine is a peptide with a low toxicity profile and a known inhibitor of GSH synthesis through inhibition of the rate-limiting enzyme γ-glutamyl synthetase. This agent that has been shown *in vitro* to increase chemotherapeutic cytotoxicity in NSCLC lines and may represent an important addition to the treatment of NSCLC (26).

In summary, the current study demonstrated higher GSH levels and GGTP enzyme activity in NSCLC compared with lung. Also, we showed increased extraction of GSH and its amino acid constituents from the pulmonary circulation by the tumor compared with normal lung. The elevation of the GGTP enzyme activity may be one mechanism underlying the increased tissue GSH levels in NSCLC. Further study is necessary to assess the role of GSH modulation in increasing the efficacy of medical management of NSCLC.

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