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IN VIVO SELECTIVE MODULATION OF TISSUE GLUTATHIONE IN A RAT MAMMARY CARCINOMA MODEL

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Abstract—Glutathione (GSH) is known to play a role in cellular sensitivity to some chemotherapeutic agents and to radiation. Depletion of cellular GSH has been demonstrated to result in enhanced toxicity of these drugs, and this approach is being explored in the clinic as a form of biochemical modulation, using the drug buthionine sulfoximine (BSO). The fact that some drug-resistant cell lines have increased glutathione levels, and that enhancing GSH concentrations in animal tissues protects against a variety of xenobiotic agents, suggest a different potential approach to improving anti-cancer therapy. We have examined the efficacy of the cysteine "pro-drug" L-2-oxothiazolidine-4-carboxylate (OTZ) at enhancing normal tissue versus tumor GSH. Animals were treated with OTZ or BSO, and the concentrations of GSH in normal tissues and tumor were measured. We found that the presence of the tumor itself decreased bone marrow GSH, but that OTZ significantly increased it in this setting. Interestingly, OTZ administration significantly depleted tumor GSH levels to the same level as did BSO. OTZ could offer a selective biochemical modulation of GSH.

Key words: glutathione, 5-oxoprolinase; L-2-oxothiazolidine-4-carboxylate (OTZ); melphalan; drug resistance

GSH§ accounts for more than 90% of total intracellular non-protein sulfhydryl and is critical in a variety of cellular defense functions including protection from toxic oxygen species and detoxification of various xenobiotics. Tumor cell GSH concentration may be among the determinants of the cytotoxicity of many chemotherapeutic agents and of radiation, and an increase in GSH concentration appears to be at least one of the mechanisms of acquired drug resistance to chemotherapy [1, 2].

Therapeutic elevation of normal cell GSH levels has also been investigated as a means to reduce the toxicity associated with a wide variety of compounds of both endogenous and exogenous origin [3, 4]. GSH may be elevated in various ways including by delivery of L-cysteine, a rate-limiting amino acid in GSH synthesis. This is difficult since cysteine is toxic, is not transported efficiently into cells, and is oxidized spontaneously at neural pH [5]. An effective method has been by administration of a "pro-drug" called OTZ [6]. This compound has been shown *in vitro* to have selectivity for normal cells as opposed to tumor cell lines [4, 7]. It has also been used *in vivo* to increase GSH concentration in the normal tissues of mice [8]. OTZ is an excellent substrate for the enzyme 5-oxo-L-prolinase, which converts it to *S*-carboxy-L-cysteine, which, in turn, spontaneously decarboxylates to yield intracellular cysteine for GSH synthesis.

To further explore this selectivity, we examined the effect of OTZ in an *in vivo* mammary cancer model developed in this laboratory. We found that OTZ increased GSH concentration effectively in some normal tissues where it may be critical in the defense against chemotherapy toxicity, while at the same time OTZ paradoxically decreased tumor GSH concentration.

MATERIALS AND METHODS

Reagents

OTZ and BSO used in the experiments reported herein were purchased from Sigma Chemical Co. (St. Louis, MO). Procysteine™ is OTZ produced commercially; it was made available to us by Free Radical Sciences Inc. (Cambridge, MA), and was used in repeated OTZ experiments. Melphalan was purchased from Sigma, and was prepared fresh in acid-alcohol solution for each use.

Experimental model

MatB 13672 is a cell line derived from a female Fischer rat mammary tumor. Cells grow both *in vitro* and *in vivo*. *In vitro* growth conditions are in α-MEM (Gibco) supplemented with 1.3% sodium pyruvate, 2.6% glutamine, 1.3% nonessential amino acids, 5% fetal bovine serum, and 100,000 U/L gentamicin. After an injection of 5×10^5 cells s.c. into 10 to 12-week-old female Fischer 344 rats, a solid mass was palpable in about 10 days. At this time, a group of animals was injected intraperitoneally with a single dose of either saline (control group), BSO (4 mmol/kg), or OTZ (5 mmol/kg). These doses were selected on the basis of previous studies demonstrating significant modulation of GSH, and on preliminary dose-finding experiments in this model (*vide infra*). Neither was found to result in any toxicity to these rats. The animals were killed 4 hr after injection, at which time samples of brain, liver, kidney, bone marrow

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§ Abbreviations: GSH, glutathione; BSO, L-buthionine-*R*,*S*-sulfoximine; OTX, L-2-oxothiazolidine-4-carboxylate; TF, tumor-free; and TB, tumor-bearing.

from limb long bones, and tumor were excised and immediately prepared for GSH determination. This schedule was chosen on the basis of both previous reports [6] and preliminary scheduling studies performed in this model demonstrating maximum effects at this time point (not shown). Because of litter-to-litter variation in the GSH concentration of some tissues, a series of separate experiments was performed using the same littermates in each, and comparisons were made within litter groups.

Tissue preparation and biochemical assays

Tissues were harvested immediately after killing the animals and were then dissected free of necrotic or hemorrhagic material at 4°. The tissues were weighed, and then homogenized with a polytron homogenizer in a 3% solution of sulfosalicylic acid. The homogenate was centrifuged at 10,000 *g* for 30 min. The remaining supernatant fluid was centrifuged at 100,000 *g* for 1 hr, and the resulting supernatant was assayed for GSH according to the technique previously described [9].

Groups of 3–5 animals were studied simultaneously under identical conditions other than the treatment (saline control, OTZ, BSO). The data presented are the results of 2–3 separate experiments. Results were analyzed using a two-tailed paired *t*-test to compare littermates given the different treatments (3–5 rats per group) in each of the experiments.

RESULTS

Table 1 confirmed that in this model OTZ effectively increases GSH in kidney and liver (44 and 65%, respectively), as has been demonstrated previously [5]. Bone marrow had not been examined previously, and we found no effect on its GSH concentration under the same conditions. Table 2 shows GSH concentrations observed in a number of tissues in TF and TB animals. It is evident that the presence of the tumor itself resulted in some changes in GSH, with a small but consistent increase in GSH in kidney (33%), but no change in liver GSH. On the other hand, the presence of the tumor resulted in a significant 25% decrease in bone marrow GSH. This is in association with a peripheral neutrophilia consistent with a colony-stimulating-factor effect on the white cell series previously described in this model [10].

The effects of a single dose of OTZ on normal tissues and on tumors in TB rats are shown in Table 3. For kidney and liver, a single injection of OTZ resulted in either no effect (kidney) or a small decrease in (12%, liver) GSH concentration. In the bone marrow, there was a 68% increase in the GSH concentration. In tumor tissue, however, there was a dramatic opposite effect, resulting in GSH depletion by OTZ.

Table 1. Effect of OTZ on tissue GSH in non-tumor bearing rats

Treatment	GSH concentration		
	Kidney (μmol/g)	Liver (μmol/g)	Bone marrow (μmol/10^6 cells)
Control	0.82 ± 0.05	3.31 ± 0.06	1.12 ± 0.05
OTZ	1.18 ± 0.03*	5.45 ± 0.02†	1.29 ± 0.04

Rats were divided into groups of 4 animals, and the treated animals received 5 mmol/kg of OTZ administered intraperitoneally. GSH was measured in tissues harvested 4 hr after treatment. Results are means ± SEM.

* $P = 0.02$.

† $P = 0.004$.

Table 2. GSH concentration in rats that were either tumor-free (TF) or had subcutaneously growing MatB mammary carcinoma (TB)

Animals	GSH concentration		
	Kidney (μmol/g)	Liver (μmol/g)	Bone marrow (μmol/10^6 cells)
TF	1.02 ± 0.06	4.32 ± 0.2	1.13 ± 0.05
TB	1.33 ± 0.12*	4.84 ± 0.2	0.87 ± 0.07†

These are the combined results of three separate experiments, in which there were 3–4 rats per group studied; therefore, N = 11 rats per group. Results are means ± SEM.

* $P = 0.002$.

† $P = 0.001$.

Figure 1 compares the effect of a single dose of OTZ versus that of BSO on normal tissue and tumor GSH concentrations in a separate series of experiments performed on a different group of animals. Although in most experiments reported BSO is given in multiple-injected doses or in the drinking water [11], even with the single dose administered here there was a dramatic depletion of tumor GSH. In addition, kidney, brain, and liver GSH were lowered. Bone marrow GSH concentration remained lower than observed in non-TB animals. Four hours after a single dose of OTZ in these normal animals, there were small decreases in liver and brain GSH, but to a much lesser extent than that produced by BSO. There was no change in kidney GSH, while bone marrow concentrations were significantly higher. The GSH depletion in tumor was not different from that observed after BSO administration. These experiments were repeated in tumor-bearing animals using Procysteine™, and the results were the same.

DISCUSSION

GSH is a tripeptide that is ubiquitous in mammalian cells, and is critical in cellular defense against a variety of toxins. GSH is synthesized from glutamate, cysteine and glycine, but availability of cysteine is the limiting factor. A series of studies have demonstrated an important role for GSH in the toxicity of a number of chemotherapy drugs and radiation in both tumor and normal tissue [1–4, 12]. The role of glutathione metabolism *in vitro* has been examined using the resistant subline murine L1210 leukemia, and also human ovarian cancer cell lines established from patients exhibiting drug resistance associated with a 2 to 3-fold increase in GSH concentration in resistant cells [2]. A number of these studies also reported elevated GSH concentration and glutathione *S*-transferase activity associated with resistance to alkylating agents. Depletion of tumor GSH can sensitize cells to anthracyclines, bifunctional alkylators, and radiation [4]. BSO, which depletes cellular GSH concentration and sensitizes tumors to melphalan in experimental systems, is currently in clinical trials [10, 13, 14].

It is noteworthy that dose-limiting toxicity of alkylating agents, such as melphalan, is in the bone marrow, so

Table 3. GSH in tumor-bearing rats treated with OTZ

Treatment	GSH concentration			
	Kidney (μmol/g)	Liver (μmol/g)	Bone marrow (μmol/10^6 cells)	Tumor (μmol/g)
Control	1.56 ± 0.1	5.36 ± 0.13	0.75 ± 0.07	1.74 ± 0.07
OTZ	1.46 ± 0.13	4.84 ± 0.12*	1.27 ± 0.11†	0.61 ± 0.06‡

There were seven animals per treatment group; animals in the experimental group were injected intraperitoneally with 5 mmol/OTZ/kg. Tissue was harvested 4 hr afterwards. Results are means ± SEM.

* $P = 0.007$.

† $P = 0.001$.

‡ $P = 0.0001$.

that one important goal is selective biochemical modulation of tumor versus bone marrow GSH. In both animal studies and two phase I clinical trials of the combination of BSO with melphalan, enhanced bone marrow toxicity has been noted and could be an important limitation [10]. Our study confirmed the effect of BSO on GSH concentrations in rat mammary cancer cells grown *in vivo*, as well as in normal tissues. The data presented here suggest that OTZ may offer the benefit of selectivity. OTZ is an ideal delivery system for cysteine, requiring only the activity of the enzyme 5-oxoprolinase, an intracellular enzyme that generates cysteine intracellularly from OTZ [15, 16]. It has been used effectively to raise GSH levels, and has been shown to result in cellular protection from a variety of toxic chemicals including chemotherapy [5, 7, 14].

Although in our model we were able to confirm the elevation of GSH after injection of OTZ (5 mmol/kg) in various tissues (liver, kidney) of normal control non-TB animals, in TB rats OTZ treatment resulted in an increase only in bone marrow GSH concentration, while paradoxically decreasing GSH in tumor to the same degree observed with BSO treatment. One possible mechanism of selectivity of OTZ may be the difference in the specific activity of 5-oxoprolinase among tissues. It has been suggested previously that normal cells have a higher 5-oxoprolinase activity than tumor cell lines *in vitro* [3, 6]. Another potential mechanism for the observed effect of OTZ on tumor GSH is the fact that OTZ serves as a competitive inhibitor of this enzyme, competing with the natural substrate 5-oxoprolinase. This could result in a depletion of glutamate for GSH synthesis, and thus shift the rate-limiting substrate from cysteine to glutamate. If tumor cells make greater use of glutamate, as has been demonstrated in some systems [15], or if the 5-oxoprolinase activity of the tumor is low, as has also been suggested for some tumor cells [7], this would be a reasonable scenario.

Another observation in our study was the impact of the presence of a tumor itself on GSH concentration in normal tissue. The mechanism of this effect is unknown; however, we have demonstrated previously in this model that there is a significant increase in peripheral neutrophils that progressively increase with tumor growth, and it was suggested that the tumor may produce or induce a colony-stimulating factor [10]. Two other studies have suggested that tumor-related or induced factors such as tumor necrosis factor α can alter the expression of enzymes such as catalase in normal tissue [16, 17]. It is therefore possible that TB animals produce and secrete a factor that can interfere with GSH metabolism in some normal tissues, explaining both the different GSH concentration in normal tissues of TB animals compared with controls and also their different response to a modulating agent such as OTZ. On the other hand, the marrow stimulation no doubt results in a change in the mar-

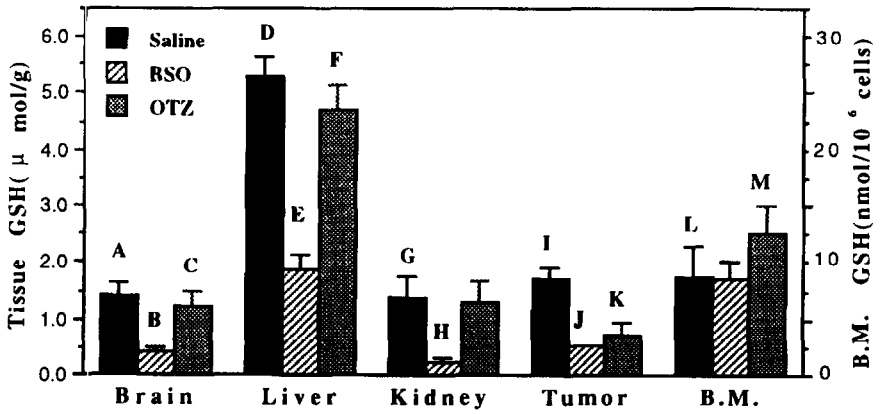

Fig. 1. Effects of BSO and OTZ treatment on the glutathione (GSH) concentration of various tissues. This figure demonstrates the results (means ± SEM, N = 3–5 animals per group) in normal tissues and tumor of tumor-bearing rats after intraperitoneal treatment with saline, BSO, or OTZ. B.M. = bone marrow. The letters are placed for statistical comparison of the results for each group: $P < 0.001$ for A:B, D:E, I:J, I:K; $P < 0.01$ for D:F, G:H; and $P < 0.05$ for A:C, L:M.

row population of cells, and this may also be reflected in changed GSH concentration observed in the mononuclear cell preparation we examined. Not enough is known at present about GSH concentration in different cell lineages and in cells at various stages of maturation to speculate on this.

Procysteine™ has undergone initial testing in humans in a phase I study done in healthy volunteers, and is undergoing study in HIV-positive subjects. [18]. There are no studies in the context of cancer therapeutics, and the *in vivo* data presented here provide a rational basis for the consideration of studying OTZ as a biochemical modulator of chemotherapy.

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