Glutamine Affects Glutathione Recycling Enzymes in a DMBA-Induced Breast Cancer Model

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Malignancy depletes host glutathione (GSH) levels to increase treatment-related toxicity and increases itself to resist the treatments. Our previous studies have shown that dietary glutamine (GLN) prevented 7,12-dimethylbenz[a]anthracene (DMBA)-induced mammary tumors through enhancing gut GSH release and reducing tumor GSH level. In addition, GSH synthesis, metabolism, and recycling are accomplished in $\gamma$-glutamyl cycle. We hypothesized that the GLN prevention might be through a differential regulation of the $\gamma$-glutamyl cycle enzymes. Female Sprague-Dawley rats were randomized into DMBA-tumor bearing, DMBA-treated, and control groups subdivided into GLN and water groups. GLN supplementation was given at 1 g/kg/day by gastric gavage. The activities and messenger RNA levels of $\gamma$-glutamyl transpeptidase (GTP), $\gamma$-glutamylcysteine synthetase (GCS), 5-oxo-L-prolinase (OPase), $\gamma$-glutamyl transferase (GTF), and glutaminase (GLNase) were determined in gut mucosa and breast tumor using specific enzyme assays and semiquantitative reverse transcription polymerase chain reaction. GLN upregulated gut GTP, GCS, OPase, and GLNase in DMBA-tumor bearing, DMBA-treated, and/or control rats; however, it downregulated these enzymes in the tumor. The paradoxical effect of GLN on key GSH recycling enzymes in the gut versus tumor suggests that dietary supplemental GLN could be used in the clinical practice to increase the therapeutic index of cancer treatments by protecting normal tissues from, and sensitizing tumor cells to, chemotherapy and radiation-related injury.

INTRODUCTION
Cancer cachexia is associated with marked depletion of glutamine (GLN) (1) accompanied by reduced glutathione (GSH) levels (2). These conditions are exacerbated by the effects of their treatments (3). Some studies have demonstrated that dietary GLN can restore the depletion and improve outcomes of cancer treatments (3,4). In some experiments, we have shown that oral GLN supplementation decreased 7,12-dimethylbenz[a]anthracene (DMBA)-induced mammary carcinogenesis by 50% to 75% (4,5). It was found that DMBA-induced breast tumor development correlated with a significant block in gut GSH release (6). We suggested that this block might lead to a decreased supply of the important antioxidant GSH at a time when it may be crucial for the prevention of oxidative damage produced by DMBA and at a place where most DMBA metabolism may be activated and further result in DNA adducts in the breast (6). It was also found that oral GLN restored the depressed gut GSH release, increased gut GSH concentration, and preserved normal gut structure (7). In addition, oral GLN significantly decreased tumor GSH level and further stimulated tumor apoptosis (8).

The epithelial cells of the gut are highly dependent on GSH, the deficiency of which leads to a marked cellular degeneration, suggesting that enhanced GSH might be of therapeutic value in protecting the gastrointestinal epithelia against toxicity associated with oxidative damage such as seen with chemotherapy and radiation (9,10,11). For example, adriamycin and methotrexate, commonly used in breast cancer treatment, promote free radical formation and decrease GSH in various organs (12,13). Accordingly, clinical studies have suggested that antioxidants in combination with chemotherapy and irradiation prolonged the survival time of patients compared to expected outcome without the antioxidant supplements (13,14). On the other hand, the resistance of the tumor cell to a variety of anticancer agents is often associated with increased GSH levels (15), indicating that reduction of tumor GSH may enhance tumor sensitivity to radiation and chemotherapy.

Intracellular GSH biosynthesis and metabolism are accomplished in the $\gamma$-glutamyl cycle (16). The $\gamma$-glutamyl cycle is composed of a series of interrelated enzymatic reactions that link the de novo synthesis and degradation of GSH, thus regulating the intracellular GSH concentration. The transfer of $\gamma$-glutamyl
moiety from extracellular GSH to acceptor amino acids at the cell membrane is catalyzed by γ-glutamyltranspeptidase (GTP) Enzyme Commission Number (EC 2.3.2.2.). γ-glutamyl amino acids formed at the outer surface of the membrane are then transported into the cell. If GLN is the acceptor, the by-products of this reaction are cystinylglycine and γ-glutamyl-glutamine. The reaction not only breaks down an extracellular GSH but also oxidizes an intracellular GSH. The γ-glutamyl amino acid is converted to 5-oxo-L-proline by γ-glutamylcyclotransferase, which is further converted to glutamate (GLU) by the enzyme 5-oxo-L-prolinase (OPase, EC 3.5.2.9.), by utilizing 1 adenosine 5′-triphosphate (ATP) molecule. GLU is 1 of the starting materials for GSH synthesis. Cystinylglycine is enzymatically split by a dipeptidase to cysteine and glycine, which are further used as substrates for GSH resynthesis. Thus, GSH is synthesized from its constituent amino acids (GLU, cysteine, and glycine) in 2 sequential, ATP-dependent, enzymatic steps, catalyzed by γ-glutamylcysteine synthetase (GCS, EC 6.3.2.2.) and GSH synthetase. Alternatively, extracellular GLN could provide GLU for GSH synthesis via the reaction of γ-glutamyltransferase (GTF, EC 2.3.2.1.) or glutaminase (GLNase, EC 3.5.1.2.). Therefore, the enzymes: GTP, GCS, OPase, GTF, and GLNase play key roles in regulating GSH synthesis and recycling.

We hypothesized that dietary GLN might affect the activity and expression of enzymes involved in GSH synthesis, thus inhibiting the DMBA-induced carcinogenesis. In this study, we examined the effect of dietary GLN on enzyme activities of GTP, GCS, OPase, GTF, and GLNase in jejunal mucosa and DMBA-induced mammary gland tumors of rats.

MATERIALS AND METHODS

Experimental Animals and Tissue Preparation

A total of 48 age-matched, female, Sprague-Dawley rats (38 days old) were purchased from Harlan Sprague-Dawley, Inc. (Indianapolis, IN). All studies were approved by the Animal Care and Use Committee at Central Arkansas Veterans Healthcare System. The rats were randomized into DMBA + Tumor (n = 16), DMBA – Tumor (n = 16), and control (n = 16) groups and further subdivided into GLN and water (H2O) groups. Thus, the experimental groups were DMBA + Tumor + GLN (n = 8); DMBA + Tumor + H2O (n = 8); DMBA – Tumor + GLN (n = 8); DMBA – Tumors + H2O (n = 8); Control + GLN (n = 8); and Control + H2O (n = 8). During the study, all rats were pair fed the defined chow (TD 96163; Harlan Sprague-Dawley Inc.) and given water ad libitum. Pair feeding was conducted by balancing chow intake among animals, which followed the idea that more chow was given to the animal that ate less, whereas less chow was given to the animal that ate more. Pair feeding is necessary to balance the chow intake among the groups to ensure an isonitrogenous and isocaloric diet because the tumor and/or the various treatments may depress food intake. Thus, all experimental rat weights were similar. The TD 96163 chow was used to avoid the effects of artificial antioxidant and variations often seen in standard chow of mineral elements and vitamins. In addition, all rats received daily either GLN (1 gm/kg/day; “AES-14,” provided by Aesgen Inc., Princeton, NJ) or H2O by gastric gavage. At age 52 days, the rats were gavaged with a 1-time dose of 100 mg/kg body weight DMBA (Sigma Chemical Co., St. Louis, MO) in 1 ml sesame oil or with 1 ml sesame oil alone for control rats. The rats in DMBA – Tumor + GLN and DMBA – Tumor + H2O, which were defined as DMBA-treated groups, were sacrificed 1 wk after DMBA application; the rats in DMBA + Tumor + GLN and DMBA + Tumor + H2O, which were defined as tumor-bearing groups, were sacrificed 11 wk after DMBA application; and the rats in Control + GLN and Control + H2O, which were defined as nontreated control groups, were sacrificed 11 wk after sesame oil application. At sacrifice, anesthesia was obtained with 50 mg/kg Nembutal (Abbott Laboratories, Stone Mountain, GA) by intraperitoneal injection. Jejunum (10 cm) was obtained through a midline incision and rinsed free of debris with saline. Mucosal scrapings were frozen in liquid nitrogen and stored at −80°C until used. Jejunum, the central of small intestine, was chosen because it is characterized as having extensive and long plicae circulares and having long and slender villi compared to the other 2 parts (duodenum and ileum) (17,18); thus, it is the main site of gut absorption of most nutrients and minerals. Moreover, the jejunum was defined as a major site of GLN absorption (19). The tumors were separated from the normal surrounding breast tissue, frozen in liquid nitrogen, and stored at −80°C until used.

GTP Activity Determination

The activity of GTP was measured using L-γ-glutamyl-3-carboxy-4-nitroanilide (glucana) as a substrate and 3-carboxy-4-nitroaniline (cana) as a product by a method described by Wahlefeld and Bergmeyer (20). The tissue was homogenized in homogenization solution (150 mM sodium chloride, 100 mM Tris/HCl, and 0.1% (vol/vol) triton X-100, pH 8) at the ratio 1:5 (wt/vol). The enzyme GTP activity was expressed as micromoles per mg protein; 1 unit of GTP activity was defined as the amount of enzyme that would catalyze the formation of 1 μmole cana per min under the conditions of the assay procedure.

GCS Activity Determination

The GCS activity determination was based on the assay method (involving ATP converting to adenosine diphosphate and inorganic phosphate) provided by Sekura and Meister (21) and the measurement method for product (inorganic phosphate) by the method of Taussky and Shorr (22). The tissue was homogenized in a homogenization solution (150 mM potassium chloride, 5 mM 2-mercaptoethanol, and 1 mM magnesium chloride) at ratio 1:5 (wt/vol). The enzyme GCS activity was expressed as microgram inorganic phosphate per mg protein.
**OPase Activity Determination**

The OPase enzyme activity was measured using 5-oxo-L-proline as a substrate and GLU as a product by the methods of Weber and Wolf (23), and the modified method for GLU measurement of Bernt and Bergmeger (24). The tissue was homogenized in homogenization solution (50 mM pH 7.2 Tris/HCl, 0.1 mM ethylenediamine tetraacetic acid, 5 mM 5-oxo-L-proline, 2 mM dl-dithiothreitol, and 0.25 M sucrose) at ratio 1:5 (wt/vol) and centrifuged at 18000 g, 4°C, for 20 min. The OPase activity was expressed as nmole GLU/h/mg protein.

**GTF Activity Determination**

The GTF activity was measured by a method described by Thorndike and Reif-Lehrer (25), which is based on the reaction GLN and hydroxylamine produces glutamylhydroxamate. The tissue was homogenized in distilled water (1:5, wt/vol). The enzyme GTF activity was expressed as pmole glutamyl hydroxamate formed/min/mg protein.

**GLNase Activity Determination**

The activity of GLNase was determined by the method, measuring the product GLU from the starting material GLN, provided by Pinkus and Windmueller (26). The tissue was homogenized in a homogenization solution (330 mM sucrose, 50 mM trizma hydrochloride, and 5 mM magnesium chloride) at ratio 1:7.5 (wt/vol). The activity of GLNase was expressed as µmole GLU/mg protein.

**Protein Concentration Measurement**

The protein concentration of each sample was measured by BioRad protein assay (Bio-Rad Laboratories, Hercules, CA).

**RNA Extraction and Semiquantitative Reverse Transcription Polymerase Chain Reaction (RT-PCR)**

The expression levels of GTP, GCS, OPase, GLNase, and GTF transcripts in gut mucosa and tumors were determined by a semiquantitative RT-PCR as described elsewhere (27,28). Briefly, total RNA isolated using RNeasy Mini Kit (Qiagen, Chatsworth, CA) was reverse transcribed using Ready-To-Go You-Prime First-Strand Beads (Amersham, Piscataway, NJ) and oligo (dT; Promega, Madison, WI). First-strand complementary DNA was used as a template for the subsequent PCR-amplification with the following oligonucleotide primers: GTP (GenBank Acc# M33821 forward 5′ TCACAACCC-CACAACCTTC, reverse 5′ AACCTGCAAATGCAAGGC), GCS catalytic subunit (GenBank Acc# J05181, forward 5′ CCAAGCAAGGCGATAACAG, reverse 5′ AGTGGCC-CCATCCCACAATCC), OPase (GenBank Acc# U24174, 5′ GATCCTGGTGTGTCGCCACCT, reverse 5′ CATGGCC-GAAGTCAAGGTCC), GLNase (GenBank Acc# BN000385, forward 5′ CTCTTGAACCTCTCGTCTC, reverse 5′ AGCCTGTGGTGGCAAAATG). A total of 35 cycles of reaction at 94°C (45 s), appropriate annealing temperature (45 s), and extension at 72°C (1 min), followed by 10 min at 72°C, were carried out in Perkin-Elmer (Waltham, MA) 2400 thermal cycler. The amount of each of the transcripts was quantified by concurrently amplifying the 18S ribosomal unit as an internal control (Ambion, Austin, TX). The area and density of the bands resulted from RT-PCR were measured using Scion Image Program for IBM (Scion Corporation). The ratio of the signals of each target gene and 18 Svedberg units (18S) ribosomal RNA (target gene/18S) was calculated individually. The results, expressed as densitometric units, were analyzed statistically.

**Statistical Analysis**

Comparisons between the groups were performed by a 1-way analysis of variance using statistical software StatView for Windows, version 4.5. All data were expressed as mean ± SE. Results with P < 0.05 were considered statistically significant.

**RESULTS**

The effects of GLN on tumor development and tumor and gut mucosa GSH levels were presented and discussed elsewhere (4–8). The data showed that GLN supplementation reduced tumor development in the DMBA-breast cancer model by 50% to 75%, and this was associated with reduced tumor weight and volume (4,5). GLN also caused a significant decrease of total GSH in the tumors by 57% (8) and a several-fold increase of GSH levels in the gut mucosa (5,7).

**GTP Activity**

In this study, DMBA and tumor presence did not have any effect on gut mucosa GTP activity in the tested groups (Fig. 1A). Dietary GLN supplementation resulted in a significant increase of the GTP enzyme activity in jejunal mucosa of normal, DMBA-treated, and tumor-bearing hosts vs. H2O (45 s), and extension at 72°C (1 min), followed by 10 min at 72°C, were carried out in Perkin-Elmer (Waltham, MA) 2400 thermal cycler. The amount of each of the transcripts was quantified by concurrently amplifying the 18S ribosomal unit as an internal control (Ambion, Austin, TX). The area and density of the bands resulted from RT-PCR were measured using Scion Image Program for IBM (Scion Corporation). The ratio of the signals of each target gene and 18 Svedberg units (18S) ribosomal RNA (target gene/18S) was calculated individually. The results, expressed as densitometric units, were analyzed statistically.

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EFFECT OF GLUTAMINE ON GUT AND TUMOR GLUTATHIONE-RELATED ENZYMES

FIG. 1. $\gamma$-glutamyl transpeptidase (GTP) activity of jejunal mucosa (A) and tumor (B). A: The glutamine (GLN)-treated groups have higher gut mucosa GTP activity in non-7,12-dimethylbenz[a]anthracene (DMBA) treated (control), DMBA treated before tumor appearance (DMBA – Tumor), and DMBA-induced, tumor-bearing (DMBA + Tumor) conditions. B: There is a significant low tumor GTP activity in GLN-treated group. The GTP activity is expressed as microunit/mg protein. ANOVA, analysis of variance.

rats (Fig. 3A). GLN had no effect on OPase activity in DMBA-treated rats without tumors or in the controls. However, GLN significantly inhibited tumor OPase activity (Fig. 3B).

GTF Activity
We found no significant effect of GLN on GTF enzyme activity in any of the experimental groups, although there was a trend toward GTF stimulation by GLN supplementation in the gut (not shown).

GLNase Activity
In our experiments, oral supplemental GLN significantly increased the GLNase activity of the gut mucosa in all cases in comparison with the controls (Fig. 4A). There was a significantly lower GLNase activity in tumors of animals that received GLN supplementation (Fig. 4B).

Effect of Dietary GLN on Messenger RNA (mRNA) Levels of GTP, GCS, OPase, GLNase, and GTF in Jejunal Mucosa and Tumor Cells
To evaluate the impact of GLN on the gene expression of GTP, GCS, OPase, GLNase, and GTF in the jejunal mucosa and tumor cells, we used relative RT-PCR (27,28). The relative expression level of each specific gene was determined by comparison with the signal obtained for 18S mRNA, and the data were statistically analyzed.

The results show that the expression levels of all of the examined genes were not affected by GLN in the control

FIG. 2. $\gamma$-glutamylcysteine synthetase (GCS) activity of jejunal mucosa (A) and tumors (B). A: Glutamine (GLN) supplementation significantly increases the gut mucosa GCS activity in non-7,12-dimethylbenz[a]anthracene (DMBA) treated (control), DMBA treated before tumor appearance (DMBA – Tumor), and DMBA-induced, tumor-bearing (DMBA + Tumor) hosts versus H2O-fed groups. There is no difference among control, DMBA-treated, and tumor-bearing groups. B: GLN significantly decreases GCS activity in tumor. The GCS activity is expressed as microgram inorganic phosphate/mg protein. ANOVA, analysis of variance.
FIG. 3. 5-oxo-L-prolinase (OPase) activity of jejunal mucosa (A) and tumors (B). A: Glutamine (GLN) enhances OPase enzyme activity in jejunal mucosa of tumor-bearing rats in comparison with the rats that received H2O. B: GLN significantly inhibits tumor OPase activity compared to H2O-treated groups. The OPase activity is expressed as nmole glutamate/h/mg protein. ANOVA, analysis of variance.

groups. In the DMBA-tumor-bearing groups, we compared GTP, GCS, OPase, and GLNase mRNA levels in jejunal mucosa and tumor of the rats that received GLN with those received H2O and found that dietary GLN significantly enhanced GTP and OPase transcripts (Fig. 5A) but not GCS and GLNase in jejunal mucosa; however, dietary GLN significantly inhibited GTP and GCS gene expression in tumors (Fig. 5B) without a significant effect on mRNA levels of the rest of the examined enzymes.

DISCUSSION

The results from this study showed that oral dietary GLN upregulated GTP, GCS, and GLNase involved in GSH synthesis and recycling in host gut under carcinogen-naive, carcinogen-treated, and tumor-bearing conditions; in contrast, dietary GLN significantly downregulated tumor GTP, GCS, OPase, and GLNase. These results are in agreement with previously established enhanced gut GSH release seen with GLN and further the inhibition of DMBA carcinogenesis (4,5). OPase was only elevated in the gut of the tumor-bearing host, which also supports enhancing gut GSH release. Meanwhile, the results also support our previous finding that dietary GLN decreased GSH concentration in the tumor (8). Furthermore, the results indicate that dietary GLN may increase gut GSH metabolism to reduce the treatment-induced toxicities and may decrease tumor GSH level to increase tumor sensitivities to the treatments.

Increased GTP activity associated with higher GSH concentration and upregulation of DNA synthesis was found to promote the growth rate of B16 melanoma cells as well as their metastatic potential in mouse liver (29). The expression of GTP

FIG. 4. Glutaminase (GLNase) activity of jejunal mucosa (A) and tumors (B). A: Oral glutamine (GLN) significantly enhances gut GLNase activity in all examined groups. B: GLN-treated group shows significantly lower tumor GLNase in comparison with H2O-treated groups. The GLNase activity is expressed in µmole glutamate/mg protein. ANOVA, analysis of variance.
has been regarded as a marker of neoplastic progression in several experimental models such as rodent skin and liver chemical carcinogenesis (30). In a clinical study of patients with breast carcinoma, Mishra et al. (31) established significantly higher blood levels of GTP in patients with metastasis vs. patients without metastasis and normal controls. Significantly higher levels of GTP have been reported also in a number of human malignant neoplasms, for example, ovary, colon, lung, and leukemia (32). The expression of GTP was suggested to participate in mechanisms of drug resistance of cancer cells (33). This study indicates that dietary GLN was able to downregulate GSH synthesis and to inhibit significantly both GTP enzyme activity and gene expression in DMBA tumors of rats. In rodents, there is a single GTP gene, and several promoters that generate different mRNA subtypes and regulate its expression. During oxidative stress, GTP gene expression is increased, suggesting a specific mode of regulation of GTP gene expression by oxidants (34).

Increased activity and expression of GCS was associated with increased GSH levels in different drug resistant human tumors. For example, human T98G glioblastoma cells were resistant to ionizing radiation and cisplatin chemotherapy (35); MCF-7 mammary cells were resistant to doxorubicin and 4-hydroperoxycyclophosphamide chemotherapy (36); and lung cancer cells were resistant to platinum drugs (37). These resistances were related to overexpressed GCS and increased GSH concentration that can contribute to the defense against oxidative damage generated by ionizing radiation and drug chemotherapy (35–37). The increased enzyme activity of gut GCS in this study was not associated with increased gut GCS mRNA levels. GCS is known to be a subject of a feedback inhibition by GSH (38). Our results indicate that GLN, through a differential regulation of GCS, could regulate GSH synthesis in a tumor-bearing host. Moreover, GLN has none of the toxic side effects of L-S,R-buthionine sulfoximine, which is the commonly used GCS inhibitor.

It has been reported that in stressed conditions, host GLU becomes a rate-limiting substrate for GSH synthesis, and OPase becomes the rate-limiting enzyme for \( \gamma \)-glutamyl cycle (39). The results from this study indicate that in DMBA-tumor-bearing host, the upregulation of OPase activity by supplemental GLN could become a major factor for maintaining the gut GSH biosynthesis and supporting the host defense system. Moreover, the results also show that the increased OPase activity by GLN may result from its effect on OPase gene expression. In addition, GLN supplementation downregulated tumor OPase enzyme activity, which further resulted in a depletion of tumor GSH that could increase tumor sensitization to anticancer treatment.

There are some alternative pathways through the reactions of GTF and/or GLNase to provide GLU as a substrate for GSH synthesis. Thus, in this study, we examined GTF and GLNase enzyme activity or gene expression.

In a tumor-bearing host, gut mucosa GLNase activity diminishes as the tumor grows because the tumor becomes the principal organ of GLN use (40,41). The results from this study showed that oral GLN supplementation enhanced GLNase activity of the gut mucosa but did not affect GLNase gene expression. This finding is in accordance with the data of James et al. (42) showing that despite the high potential for GLN metabolism, the intestines have little or no synthesizing capacity. GLN deprivation of tumor cells could be a therapeutic goal. Several investigators have examined antitumor activity of GLNase such as bacterial GLNase (43) and Cu-GLNase (44). However, most of them have the disadvantage of limited efficacy and/or toxicity (45). The results of this study suggest that supplemental GLN itself can be a better tumor GLNase inhibitor because GLN differentially affects GLNase activity in host...
gut vs. tumor; moreover, GLN supplementation is easy, non-toxic, and inexpensive.

GTF is considered responsible for the formation of the hydroxamate of GLU from GLN and hydroxylamine. It has been suggested that this enzyme deamidates (e.g., hydrolyzes) the substrate GLN and produces γ-glutamyl radical, which can be transferred to acceptor molecules. When this enzyme exhibits hydrolytic activity, it acts like GLNase to produce GLU (46). However, no significant difference in the GTF activity was found in gut mucosa and tumor among tested groups. The results indicate that the mechanism by which GLN affects the GSH synthesis is not associated with this enzyme activity.

The lack of the consistency between the enzyme activities and mRNA levels of GCS, OPase, and GLNase might indicate metabolic regulation of gene expression, although further studies are required.

The possible explanations for the paradoxical effect of GLN on GSH metabolism of tumor and host tissues were summarized in a review paper by Savarese et al. (3). One possibility is that a local increase in GLU concentration by GLN inhibits GSH transport into tumor mitochondria but not the mitochondria of normal cells (47). Our group’s hypothesis (48,49) is that tumor cells have a relatively more acidic intracellular environment compared to normal cells, thus inactivating the pH-sensitive OPase. In normal cells, this block can be overcome by providing excess GLN, which acts as a γ-glutamyl acceptor, forming γ-glutamyl-glutamine dipeptide and upregulating the GLNase. In contrast, these enzymes cannot be upregulated in the tumor cells, and the net result is that intracellular GSH levels are depleted in tumor but not in normal tissue.

In conclusion, the results from this study indicate that dietary GLN paradoxically modulates GSH metabolism of normal vs. tumor cells in a tumor-bearing host. Therefore, GLN supplementation selectively targets cancer while protecting the host and could be used to increase the therapeutic effectiveness ofanticancer agents.

ACKNOWLEDGMENTS

This work was supported by VA Merit Review Award to V. S. Klimberg.

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