Effect of Glutamine on Gut Glutathione Fractional Release in the Implanted Tumor Model

Yihong Kaufmann and V. Suzanne Klimberg

Abstract: Cancer and its treatments cause a marked depletion of glutamine (GLN). However, dietary GLN can restore this loss and improve the outcomes of the treatments. The reasons behind this need to be investigated. GLN is suggested to involve in glutathione (GSH) synthesis. Fast-growing tumors alter gut GLN metabolism, but the effect of tumor growth on gut GSH release remains unknown. We hypothesized that gut GSH release would decrease in the tumor-bearing host and this downregulation would be antagonized by supplemental GLN. Female Fisher-344 rats were randomized to the groups: GLN + TUMOR, Frearline (FA) + TUMOR, GLN + SHAM, and FA + SHAM. The rats were implanted with MTF-7 mammary tumors as tumor-bearing groups, whereas the rats were sham operated as control groups. The rats were pair fed chow, gavaged with 1 g/kg/day GLN or an isonitrogenous FA. Tumor growth, blood and gut mucosa GLN, glutamate, and/or GSH were measured. The gut extractions, defined as the difference of concentrations across the gut, were calculated. Supplemental GLN enhanced the gut GLN uptake and GSH release with tumor growth and significantly increased blood and gut mucosa GLN and/or GSH concentrations. Our results demonstrate the important antioxidant role of GLN and thus may have significant implications in nutritional immune modulation in cancer patients.

Introduction

Advanced malignant diseases can cause a marked loss of glutamine (GLN) in the host. This condition is exacerbated by the effects of their treatments (1). The deficiency of GLN in the host may cause cancer cachexia (2). Some studies have demonstrated that dietary GLN can restore this depletion and improve outcome (3-5). However, concern exists over providing supplemental GLN to the patient with cancer because it may stimulate tumor growth because GLN is the major respiratory fuel utilized by most rapidly growing tumors (6). Therefore, the benefit of supplemental GLN in patients with cancer is controversial. It is important to elucidate the mechanisms of GLN actions.

It has been suggested that GLN may incorporate into the body’s most important antioxidant glutathione (GSH) synthesis (7). Welbourne (8) found that GLN became rate-limiting substrate for GSH synthesis in rat kidney not under normal conditions but under oxidative stress. Hong et al. (9) demonstrated that the administration of supplemental GLN after lethal hepatic injury maintained liver GSH levels. Klimberg (10) previously showed that oral GLN recovered the depressed GSH levels in lung, liver, kidney, heart, gut, and muscle after radiation and chemotherapy. Thus, GLN might maintain or enhance GSH production to protect the host against cancer and its treatments.

GLN is utilized at high rate in the gut where the glutaminase content is very high (11). The gut also is an initiator of the interorgan GLN cycle (12). Furthermore, our lab previously discovered that the gut was a GSH producer, and supplemental GLN enhanced the gut GSH production at normal condition in the rat model (13). Thus, GLN might increase the gut GSH release and further benefit the host with cancer and its treatment.

It has been known that fast-growing tumors interfered with interorgan GLN cycle, which is to mobilize GLN stores, and further affect GLN metabolism (14). Moreover, it has been demonstrated that supplemental GLN maintained the normal gut GLN metabolism disrupted by tumor growth (10,14). However, the effects of tumor growth on gut GSH metabolism and gut GSH production have not been studied.

Therefore, this study was designed to examine the effects of dietary GLN on blood and gut mucosa GSH concentrations, gut GSH release, and tumor growth in tumor-bearing rats. We hypothesized that in the tumor-bearing host, the gut GSH fractional release that is proportional to production would decrease as a result of the known decrease in gut GLN metabolism; meanwhile, the blood and gut mucosa GSH levels would decrease as well, and this GSH downregulation would be antagonized by supplemental GLN.

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Materials and Methods

Animal Preparation and Diets

Female Fisher-344 rats weighing approximately 160 g were obtained from Harlan Sprague-Dawley, Inc. (Indianapolis, IN). All studies were approved by the Animal Care and Use Committee at Central Arkansas Veteran’s Healthcare System. The rats were individually maintained in standard cages in the animal care facility and were subjected to alternate 12-h periods of dark/light cycle. During the study period, the rats were pair fed a predefined diet of chow (Purina Rodent Chow 5001, Purina Inc., St Louis, MO) and given water ad libitum. Pair feeding is necessary to ensure the similar chow intake among groups because the tumor and/or the various treatments may depress food intake. Pair feeding was conducted by balancing chow intake among animals, which followed the idea: more chow was given to the animal that ate less, whereas less chow was given to the animal that ate more. In addition, animals were randomized to receive GLN (Sigma Chemical Co., St. Louis, MO) at 1 g/kg/day or an isonitrogenous amount of Freamine (FA; McGaw, St. Louis, MO) by gavage. Fresh daily made 3% GLN aqueous solution was used as GLN supplementation; 10% FreAminex® III (amino acid injection) was used as FA gavage. Animals were gavaged 3 times a day, and the maximum gavage volume kept below 4 cc to prevent aspiration and distress. FA is a mixture of essential and nonessential amino acids, which does not contain GLN. The chow contains 3% of its protein as GLN. Nitrogen intake as GLN for the GLN-supplemented animals averaged 0.25 g of nitrogen as GLN per kg/day vs 0.06 g of nitrogen as GLN per kg/day in FA-fed animals (from chow). This allowed comparison of GLN-supplemented rats with protein-supplemented rats but not GLN-deficient rats. Diets were isonitrogenous and isocaloric.

Tumor Implantation

Female Fisher-344 rats were taken to the operating room and anesthetized with nembutal (50 mg/kg; Abbott Laboratories, Stone Mountain, GA) via intraperitoneal. One side flank was shaved. A small subcutaneous incision was made aseptically. Approximately 2 × 2 × 2 mm cube of MTF-7 mammary tumor was transplanted into the wound. The incision was closed using a single 3.0 prolene suture. The rats were allowed to recover in a recovery chamber and then returned to the colony. The tumor was obtained from a single tumor-bearing donor whose tumor was induced by injecting MTF-7 cell line in unilateral flank. This MTF-7 tumor model has been well characterized and is a clone from 13762NF rat mammary adenocarcinoma. It is fast growing, metastasizes, and never regresses spontaneously (15). This model has been used previously by this laboratory to study tumor–host metabolism interactions (16).

Study Procedure

On Day 0 of the study, 34 rats were randomized into 1 of the 4 groups: GLN + TUMOR (GLN + TUM, n = 9), FA + TUM (n = 9), GLN + SHAM (n = 8), and FA + SHAM (n = 8). Animals received either TUM implantation (MTF-7) or SHAM operation. Animals were gavaged either GLN or FA 3 times a day as described for 2 wk. On day 15 of the study, the animals were sacrificed 1 at a time alternating among the study groups starting at noon. All rats were weighed and anesthetized with Nembutal (50 mg/kg; Abbott Laboratories, Stone Mountain, GA). Under sterile conditions, a midline incision was made, and the rats were heparinized by injecting heparin sodium (1000 units/ml; Baxter, Deerfield, IL) into the artery. Arterial and portal blood was withdrawn from the aorta and portal vein using a 25-gauge needle attached to a 1-ml syringe. Blood was processed for GLN, glutamate (GLU), and GSH content. Jejunum (10 cm) was collected from which the mucosa was scraped and assayed for intracellular gut GSH content. Tumor was surgically removed and weighed.

Processing of Samples/Analytical Procedure

GLN and GLU Determination

An aliquot of heparinized whole blood was mixed with equal volumes of cold 10% perchloric acid (Aldrich Chemical Co., Milwaukee, WI) and then vortexed and centrifuged at 5 °C at 3000 g for 15 min (Sorvall Model RC5, Thermo Fisher Scientific, Inc., Waltham, MA). The supernatant was removed and neutralized with an equal amount of cold 0.48 M potassium phosphate. This was again vortexed and centrifuged at 5 °C at 3000 g for 10 min. The supernatant was removed and kept frozen at −20°C for later determination of GLN and GLU concentration by the microanalytical method described by Bernt and Bergmeyer (17).

GSH Determination

An aliquot of heparinized whole blood was immediately mixed with equal volumes of 10% 5-sulfosalicylic acid (Sigma Chemical Co., St. Louis, MO) and then vortexed and centrifuged at 5 °C at 3000 g for 10 min. The supernatant was removed and kept frozen at −80 °C. This sample was used to determine GSH content via a standard enzymatic recycling procedure as described by Tietze (18) and modified by Anderson (19). To determine GSH disulfide (GSSG) content, an aliquot of the previous supernatant was mixed with 2-vinyl pyridine (Aldrich Chemical Co., Milwaukee, WI) and triethanolamine (Sigma Chemical Co., St. Louis, MO) and assayed via the method of Griffith (20).

The gut tissue was weighed, homogenized in 5% 5-sulfosalicylic acid (Sigma Chemical Co., St. Louis, MO), and then centrifuged at 5°C at 3000 g for 15 min. The supernatant was removed and kept frozen at −80°C. This sample was used to determine GSH content as described previously.
Gut GLN, GLU, and GSH Extraction

Using the data from arterial and portal GLN, GLU, and GSH contents, the gut GLN, GLU, and GSH extractions were calculated using the following equation:

\[
\text{Extraction(\%)} = \frac{[\text{GLN, GLU or GSH}]_{\text{arterial}} - [\text{GLN, GLU or GSH}]_{\text{portal}}}{[\text{GLN, GLU or GSH}]_{\text{arterial}}} \times 100.
\]

A positive extraction is proportional to fractional uptake. A negative extraction is proportional to fractional release.

Calculations/Statistical Analysis

All data are expressed as mean ± SE. Differences between means were considered significant at the \( P < 0.05 \) level using analysis of variance. Statistical analyses were performed using StatView II (version 4.5, Abacus Concepts, Inc., Berkeley, CA).

Results

Nutrition

There was no significant difference in the mean body weight among groups (data not shown). All rats gained weight during the study period (Table 1). Due to the pair feeding, chow intake was similar in all groups (data not shown). Total calorie and nitrogen intakes from chow and gavage were not significantly different between GLN-fed and FA-fed groups. For these groups, the diet was isonitrogenous and isocaloric.

Tumor Growth

At the beginning of the study, all initially implanted tumors were similar. At the end of the study, the average tumor weight in FA-fed animals was almost doubled in comparison with GLN-fed group (Fig. 1). All tumors were less than 5% of total body weight.

Arterial GLN, GLU, and GSH Concentration

As expected, the arterial GLN levels were significantly higher in GLN-fed than FA-fed rats in the TUM group and SHAM group, and there was a significantly lower GLN level in the tumor-bearing animals fed with FA compared to the FA-fed animals without tumors. The arterial GLU levels were significantly higher in GLN-fed than FA-fed animals in both

**Table 1. Initial and Final Body Weight (BW)**

<table>
<thead>
<tr>
<th></th>
<th>Initial BW (gm)</th>
<th>Final BW (gm)</th>
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<tbody>
<tr>
<td>GLN + SHAM</td>
<td>159.6 ± 0.9</td>
<td>166.9 ± 1.4</td>
</tr>
<tr>
<td>FA + SHAM</td>
<td>156.6 ± 1.9</td>
<td>164.1 ± 1.8</td>
</tr>
<tr>
<td>GLN + TUM</td>
<td>158.1 ± 2.3</td>
<td>163.8 ± 2.7</td>
</tr>
<tr>
<td>FA + TUM</td>
<td>160.8 ± 3.2</td>
<td>167.8 ± 3.0</td>
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Abbreviations are as follows: GLN, glutamine; FA, Freamine; TUM, tumor.

**Figure 1.** Final tumor weight. The average tumor (TUM) weights in glutamine (GLN)-fed and Freamine (FA)-fed groups are demonstrated. Supplemental GLN group has significantly less TUM weight than the FA-fed group (\( P < 0.05 \)). The TUM weights are expressed in milligrams. ANOVA, analysis of variance.
Arterial glutamine (GLN), glutamate (GLU), and glutathione (GSH) concentration. The GLN, GLU, and GSH contents in arterial blood are shown. Arterial GLN, GLU, and GSH levels are significantly lower in the tumor (TUM)-bearing animals fed with FA ($P < 0.05$). GLN supplementation significantly increases arterial GLN, GLU, and GSH levels in both animals with TUMs and without TUMs compared to Freamine (FA) supplementation ($P < 0.05$). All the concentrations are expressed in $\mu$mol/l. ANOV A, analysis of variance.

Figure 2. Arterial glutamine (GLN), glutamate (GLU), and glutathione (GSH) concentration. The GLN, GLU, and GSH contents in arterial blood are shown. Arterial GLN, GLU, and GSH levels are significantly lower in the tumor (TUM)-bearing animals fed with FA ($P < 0.05$). GLN supplementation significantly increases arterial GLN, GLU, and GSH levels in both animals with TUMs and without TUMs compared to Freamine (FA) supplementation ($P < 0.05$). All the concentrations are expressed in $\mu$mol/l. ANOV A, analysis of variance.

tumor-implanted and control groups. The FA-fed animals with tumors had significantly lower arterial GLU concentration than those without tumors. Similarly, the significantly higher arterial GSH levels were determined in GLN-fed group vs. FA-fed group with tumors or without tumors, and there was a significantly decreased arterial GSH concentration in tumor-bearing animals with FA gavage in comparison with FA-fed control (Fig. 2). In addition, the ratios of GSH and glutathione disulfide (GSSG) were calculated. GLN-fed animals had significantly higher GSH:GSSG ratios than FA-fed animals in both SHAM and TUM groups. Furthermore, the tumor-bearing rats, which received FA, had significantly lower GSH:GSSG ratios than the control (FA-fed without tumor group; Fig. 3).

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Figure 3. Arterial glutathione (GSH) and glutathione disulfide (GSSG) ratio. The GSH:GSSG ratios in arterial blood are shown. The ratios are significantly high in the tumor (TUM)-bearing and non-TUM-bearing groups with glutamine (GLN) gavage ($P < 0.05$), whereas the ratio is significantly low in the TUM-bearing group without GLN gavage ($P < 0.05$). FA, Freamine; ANOVA, analysis of variance.
Gut GLN, GLU, and GSH Extraction

Gut GLN, GLU, and GSH extractions were calculated using arterial and portal blood GLN, GLU, and GSH concentrations. A positive gut GLN extraction was seen in all experimental groups. In both TUM and SHAM groups, the GLN-supplemented animals had nearly 50% more GLN uptake than the FA-fed animals. The gut GLU extraction had the similar range and pattern as the gut GLN extraction. A negative gut GSH extraction (fractional release) was shown in all the groups. There was a significantly increased GSH release in GLN-fed group compared to FA-fed group with and without tumors. In the tumor-bearing group, FA gavage significantly decreased the gut GSH release; however, GLN supplementation increased the gut GSH release and reached the range seen in the control group (Fig. 4).

Gut Mucosa GSH Concentration

There was a significant gut mucosa intracellular GSH level reduction in the FA-fed vs. GLN-fed group. The GLN-supplemented animals with implanted tumor had higher gut GSH concentration than FA-fed animals and even reached the normal range that was shown in the control FA + SHAM group. There was a lower gut GSH content seen in tumor-bearing group that were FA fed but not significantly compared to the FA + SHAM group (Fig. 5). In addition, a significantly higher ratio of GSH and GSSG was found in GLN-fed rats vs. FA-fed rats in both SHAM and TUM groups. A significantly lower GSH:GSSG ratio was seen in TUM group vs SHAM group for both GLN-fed and FA-fed animals (Fig. 6).

Discussion

Under normal circumstances, GLN is classified as a nonessential amino acid. However, many stressful or catabolic states, such as progressive tumor growth, alter the interorgan exchange of GLN by net skeletal muscle breakdown and negative nitrogen balance and result in dramatically depleting GLN in all tissues (10). GLN consumption exceeds its production. Under these circumstances, GLN is reclassified as a conditionally essential amino acid (14). Thus, it has been suggested that supplemental oral GLN could restore the depletion in the host and provide energy and substrate to the host against tumor growth during such conditions (14). Indeed, in this study, we have investigated the effect of supplemental oral GLN on tumor development using an implantable MTF-7 tumor model. Once again we have confirmed our previous findings that the tumor growth was significantly reduced in GLN-fed experimental animals. The average tumor weight in FA-fed control animals was double than that in the GLN-fed animals.

GLN is a multifunctional amino acid and is actively transported and metabolized in nearly every tissue (21). GLN is suggested to be a precursor of GSH (7). GLN may provide the glutamic acid needed for the in vivo synthesis of the antioxidant GSH. GSH is a ubiquitous thiol-containing tripeptide and has many diverse biologic functions. GSH
Figure 5. Intracellular gut glutathione (GSH) concentration. The intracellular gut GSH contents are shown. Oral glutamine (GLN) significantly increases the intracellular gut GSH concentration ($P < 0.05$). Tumor (TUM) growth decreases the gut GSH content but does not reach significance. The concentrations are expressed in $\mu$mol/g tissue. FA, Freamine; ANOVA, analysis of variance.

Figure 6. Gut glutathione (GSH) and glutathione disulfide (GSSG) ratio. The GSH:GSSG ratios in gut mucosa are shown. There are significantly high ratios in both glutamine (GLN)-fed groups with tumors (TUMs) and without TUMs ($P < 0.05$). There are significantly low ratios in both GLN- and Freamine (FA)-fed groups with TUM compared to without TUM ($P < 0.05$). ANOVA, analysis of variance.

as an antioxidant participates in detoxification and protects the organism from oxidative injuries by conjugating electrophilic/oxidizing substances. GSH as a nonantioxidant modulates diverse cell redox signal transduction, cell proliferation, and immune response (22,23). GLN may prevent tumor growth through alteration of GSH metabolism. In this study, we examined the arterial GLN, GLU, and GSH concentrations. Oral GLN significantly elevated arterial GLN, GLU, and GSH concentrations under the tested conditions. Tumor growth significantly reduced arterial GLN, GLU,
and GSH levels; however, supplemental GLN restored the depletion in the host and brought the arterial GLN, GLU, and GSH levels up to the normal values seen in the control group. The similar patterns in increased GLN, GLU, and GSH levels by oral supplemental GLN support the hypothesis that GLN might act as a precursor of GSH synthesis via providing GLU, which is one of constituent amino acids for GSH synthesis.

The gut has its traditional role in nutrient digestion and absorption. The gut also plays a primary role in metabolic, immunologic, and barrier functions. In addition, the gut is a key organ when the host responses to the critical illness and acts both as a barrier to toxicity and as a substrate processing station for the liver and other organs (24). Supplemental GLN supported intestinal mucosal metabolic functions and helped maintaining the normal morphometrics of the small intestinal mucosa as well as upregulated gut immune function in both nonstress and stress (cancer, radiation, and chemotherapy) (4,5,25). Subsequently Cao and coworkers (13) first reported that the gut was also a major releaser of GSH, and the gut GSH release was increased by GLN supplementation in the normal status. This study has determined the gut GLN, GLU, and GSH extractions in a tumor-bearing animal model. We made the same assumption as the previous one that the positive or negative extraction represents uptake or production, respectively, because it is unlikely that GLN or FA diets would have had any effect in the blood flow. A previous study showed that there was no difference in blood flow between GLN-enriched- and GLN-free-fed animals (3).

It was found that the gut GLN and GLU extractions were very similar; they both were positive and located in the same range. Because the positive extraction is proportion to uptake, the results indicate that the gut up took GLN and GLU. On the contrary, all experimental groups had negative gut GSH extractions, which are proportion to release. The gut released GSH. Oral supplemental GLN almost doubled the GLN utilization by the gut and significantly increased the gut GLU uptake and the gut GSH fractional release compared to the non-GLN-gavaged group in the tumor-bearing animal model. Moreover, the tumors were significantly decreased the gut GSH release in FA-fed animals. However, this did not affect in the GLN-fed animals. Again, the GLU results indicate that GLN may affect GSH metabolism via GLU intermediate. In addition, the intracellular gut GSH content was measured in this study; the results showed that GLN significantly increased the gut intracellular GSH level. It has been suggested that the epithelial cells of the gut are highly dependent on GSH; thus, enhanced GSH might be of therapeutic value in protecting the gastrointestinal epithelia against toxicity associated with oxidative damage such as seen with cancer, chemotherapy, and radiation (26).

GSH is the major thiol-disulfide redox buffer of the cell and has a key importance in the protection of cells from oxidative injuries (27). The GSH:GSSG ratio represents the availability of GSH to protect against oxidative reactions and the generation of GSSG from oxidative reaction and thus reflects the balance of pro-oxidants and antioxidants (28). Therefore, the GSH:GSSG ratio is an index of oxidative stress (27,28). In addition, the clinical studies have demonstrated that the GSH:GSSG redox state could be used as a marker of oxidative stress in humans (28). This study showed that the GSH:GSSG ratios of arterial blood and gut mucosa were significantly increased as a result from oral GLN supplementation. These results indicate that oral GLN significantly reduced the oxidative stress in circulation and host tissue (gut) in both control and tumor-implanted rats. Tumor presence caused a significantly higher oxidative stress in the blood and gut as indicated by the decreased GSH:GSSG ratio in tumor-bearing rats fed with FA in comparison with FA-fed rats without tumors. The oxidative state of tumor-bearing, GLN-fed rats, however, remained similar as in the control group. These results indicate that oral GLN prevented tumor growth through reducing oxidative stress.

In conclusion, the results from this study support our hypothesis that oral supplemental GLN could prevent the falls in the gut fractional release of GSH as well as in the arterial blood and gut tissue GSH levels associated with tumor growth. We have demonstrated that the gut, which is the major organ of GLN utilization, is also a major site of the GSH fractional releaser even in the tumor-bearing host. Moreover, our study shows that oral GLN supplementation replenishes host GLN and GSH stores and enhances gut GSH production without stimulating tumor growth. In contrast, our previous study showed that oral GLN decreased GSH level in tumors (29). Together, both findings suggest that oral GLN through its differential effect on host and tumor GSH synthesis could protect normal tissues from oxidative damages while at the same time increase oxidative stress in the tumor, which would be more sensitive to its treatments. Our results demonstrate the important role of GLN in the prevention of oxidative stress via alternating GSH metabolism. Therefore, they may have significant implications in nutritional immune modulation in cancer patients.

Acknowledgments and Notes

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