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Does Glutamine Supplementation Increase Radioresistance in Squamous Cell Carcinoma of the Cervix?

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Objective. **Glutamine is proposed to protect bowel from radiation. However, glutamine may decrease cancer's radiosensitivity. We evaluate glutamine's effect on the growth rate and radiosensitivity of two cervical carcinoma cell lines** *in vitro.*

Methods. **HeLa and CaSki cells were seeded at 3000 cells/well in glutamine-free medium. An increasing amount of glutamine (0.4, 10, and 20 mM) was added to the respective plates, incubated, and irradiated with a single fraction of 0.5, 1, 3, and 6 Gy. Using a growth inhibition assay and photometric analysis, the viable cells were counted on day 8. Cell counts represent a mean ± standard deviation from six experiments and are expressed in 10^3 cells. Analysis of variance was performed.**

Results. **In nonirradiated HeLa plates, absence of glutamine results in 5.7 ± 1.2 cells/well. Addition of glutamine at 0.4, 10, and 20 mM to nonirradiated cells significantly ($P < 0.0001$) increased growth to 79.1 ± 10.0, 122.5 ± 9.0, and 114.3 ± 13.9 cells/well, respectively. In culture plates irradiated with 6 Gy, HeLa cells supplemented with 0.4, 10, and 20 mM of glutamine showed lower cell counts ($P < 0.008$). A similar significant growth suppression at 6 Gy in comparison to 0.5, 1, and 3 Gy was observed ($P < 0.01$). CaSki cells showed similar patterns.**

Conclusions. **Growth of HeLa and CaSki cells** *in vitro* **requires a minimum of 0.4 mM of glutamine in the medium. Supraphysiologic glutamine concentration does not increase tumor growth or radioresistance. Glutamine should be evaluated further as a potential bowel radioprotector.**

INTRODUCTION

The relationship between radiation dose and tumor cytotoxicity provides the foundation for the most effective radiation dose in the treatment of cancer. However, a major limitation to radiation dose escalation is the increasing injury to normal tissue. Gastrointestinal toxicity has been one of many acute complications that limits radiotherapy dose. Therefore, an agent that may selectively protect normal tissue—but not tumor cells—may decrease the radiation morbidity while enhancing tumor control.

Glutamine appears to be a promising agent because it protects the gut from acute radiation injury. This nonessential amino acid, which serves as primary fuel to enterocytes [1] and lymphocytes [2], plays a crucial role in maintaining intestinal wall integrity and cellular immunity. In patients with severe injury, tissue demand for glutamine may exceed supply. This glutamine deficit results in gut atrophy and immunosuppression. Subsequently, bacteria may translocate from the gut lumen into the systemic vasculature and cause sepsis. Providing supplemental glutamine to bone marrow transplant patients has been shown to reduce infection rate, mortality, and hospital stay [3, 4].

Malnutrition is a common problem in gynecologic oncology patients. It is very likely that any added stress (surgery, chemotherapy, or radiation) may cause a glutamine deficit in these patients. This may explain the high incidence of acute enteritis in cancer patients treated with abdominopelvic radiation. The enterocytes that line the intestine may suffer from an inadequate glutamine supply. Studies in rats that received whole abdominal radiation showed that supplemental glutamine protects the intestine from acute radiation injuries [5–7]. In contrast, rats fed with regular chow suffered from a high episode of diarrhea. Additionally, jejunal biopsy of the rats fed with regular chow revealed denuded intestinal villi compared with normal mucosal histology in those receiving glutamine. This animal study suggested a potential role for glutamine as a bowel protector from acute radiation injuries.

However, glutamine may also enhance tumor growth and increase tumor resistance to radiation therapy [8]. Souba measured arterial and venous levels of glutamine in rats with sarcoma and found a marked glutamine consumption by the tumor [9]. Similarly, *in vitro,* the HeLa cell line utilizes glutamine for 50 to 98% of its aerobic energy [10]. Therefore, the

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purpose of this study is to evaluate the effect of glutamine on the growth rate and radiosensitivity of two cervical squamous cell carcinoma cell lines *in vitro.*

MATERIALS AND METHODS

HeLa and CaSki, human cervical carcinoma cell lines (American Type Culture Collection, Frederick, MD), were propagated in glutamine-free DME-F12 and glutamine-free RPMI 1640 (Gibco, Grand Island, NY), respectively. These media were supplemented with 10% fetal bovine serum, antibiotics, and antimycotics. The cells were harvested using trypsin and EDTA and centrifuged at 1200 RPM for 3 min. The cell pellets were resuspended in fresh medium and counted using a hemocytometer and vital stains. All harvests were ≥95% viable.

The cells were then grown in a 96-well, flat-bottomed microtiter tissue culture plate (Owens Corning, Toledo, OH) at 3000 cells/well. Twenty-four hours later, the media (200 μL/well) were aspirated and the wells were washed with 200 μL phosphate-buffered saline per well. Then, the media with increasing amounts of glutamine (0, 0.4, 10, and 20 mM) were added to their respective cells and plates. These media were prepared by obtaining basic medium (containing no glutamine) and adding commercial glutamine (Gibco) at a specific amount. The cells were allowed to attach to the bottom of the well and incubate in the new media for 24 h.

The next day, the cells were irradiated using fractions of 0.5, 1, 3, and 6 Gy using a 662 KeV self-contained Cesium-137 irradiator (Model Mark I-30, Shepherd & Associates, San Fernando, CA) at a dose rate of 2.4 Gy/min. During irradiation, the cells were kept at room temperature for less than 30 min. A set of plates with specified glutamine concentrations was not irradiated and served as controls. The medium in each plate was then changed for fresh medium containing the respective glutamine concentration daily.

At each time point (at 5 P.M.), media were drained from all wells (both time point and nontime point), and the cells were washed with phosphate-buffered saline. Then, fresh media were used to replace the 24-h-old media at each respective glutamine concentration. Each day, a set of six wells with different media and radiation plates was drained and stained. Cell counts were done daily to rule out possible density inhibition from growing cells in the microtiter tissue culture plates.

The amount of viable adherent cells was determined by the method of Yamamoto *et al.* [11]. Briefly, after aspiration of the phosphate-buffered saline, the cells were stained with 1% crystal violet, washed, and dissolved in 95% ethanol/40 mM hydrochloric acid. The absorbance at 595 nm for each well is determined with a Bio-Rad 3550 microplate reader (BioRad, Hercules, CA). Absorbance results were expressed as cell number by comparing absorbance of each well with a standard curve prepared from known number of cells. Cell counts represent a mean ± standard deviation from six experiments and are expressed in 10^3 cells. Analysis of variance (ANOVA) was performed with post-hoc Scheffe multiple comparison at 0.01 level of significance for the eighth day cell counts.

RESULTS

HeLa and CaSki cells appear to grow from day 1 to day 8 (last day of experiment) in the presence of glutamine. The growth of the cells did not exhibit evidence of density or contact inhibition.

HeLa cell counts. The cell count at day 8 was significantly different with various levels of glutamine (0 mM compared to 0.4 mM compared to the 10 and 20 mM) supplementation across several levels of irradiation (two-way ANOVA test of interaction; $P < 0.0001$). There is a direct correlation between cell growth and glutamine supplementation from only the 0 and 0.4 mM concentrations. Further glutamine supplementation did not increase cell counts. At all levels of irradiation, absence of glutamine results in lower cell counts at day 8. However, in the cases of glutamine supplementation, irradiation at 6 Gy results in lower cell counts than at all other levels of irradiation (Fig. 1). Scheffe multiple comparisons confirmed meaningful differences resulting from these analyses.

CaSki cell counts. The cell counts at day 8 were significantly different comparing levels of glutamine (0 mM compared to 0.4 mM compared to the 10 and 20 mM) across the various levels of irradiation (two-way ANOVA test of interaction; $P = 0.008$). Comparing cell growth across glutamine levels for each level of irradiation shows some level of significant difference in each of these five one-way ANOVA tests ($P < 0.0001$ each); however, differences across radiation levels for each level of glutamine are not uniformly significant. There are no significant differences in CaSki cell counts at day 8 among levels of irradiation when 0 or 0.4 mM of glutamine are added. When 10 or 20 mM of glutamine are added, cell counts at 6 Gy are significantly different for 0.5 or 3 Gy, but not for 0 or 1 Gy. Regardless of the level of radiation, the cell count without glutamine is lower than cell counts with glutamine—with no differences among the nonzero glutamine groups (Fig. 2). This pattern of differences is similar to that seen in HeLa cells.

In conclusion, both HeLa and CaSki cell lines demonstrated a significant increase in growth associated with the addition of 0.4 mM glutamine or greater concentrations in nonirradiated cultures. Although the addition of glutamine in excess of 0.4 mM to irradiated cultures increased growth rate over nonirradiated cells with no glutamine present, both CaSki and HeLa cell lines showed radiosensitivity that did not change with increasing doses of glutamine.

DISCUSSION

Glutamine has been recategorized recently as a conditionally essential amino acid [12, 13]. Since it serves as a primary fuel

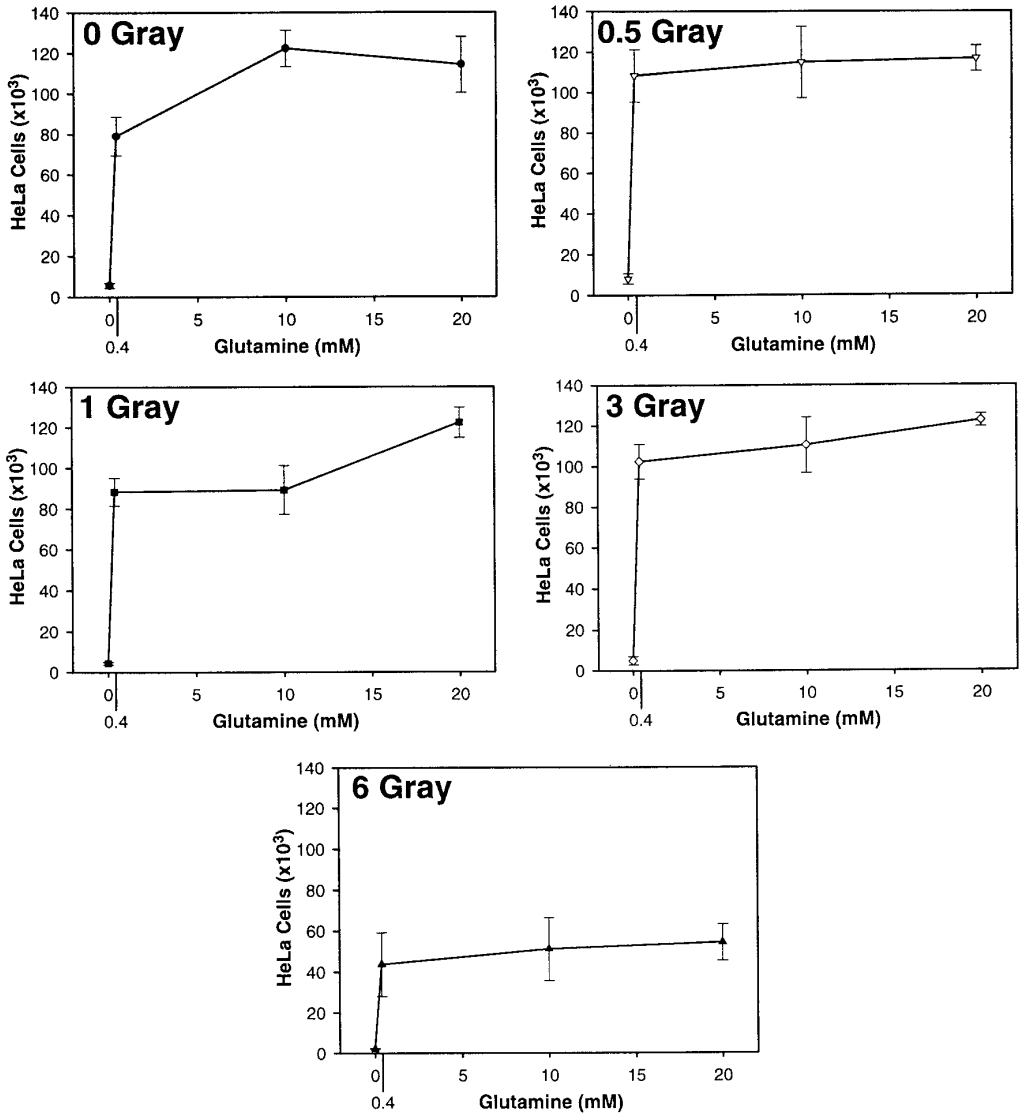

FIG. 1. HeLa cell counts at day 8 ($\times 10^3$). The cells were incubated with varying concentrations of glutamine and irradiated at four different radiation doses (0.5, 1, 3, and 6 Gy) and one control (0 Gy). Cell counts values are mean ± standard deviation of six replicates.

for the gut, glutamine has been advocated as an adjuvant agent to rescue bowel from acute radiation injury. Studies in rats [5, 6, 14] have shown that a glutamine-rich diet protects intestinal cells from radiation-induced injuries.

However, glutamine may also promote tumor cell growth [8]. There was a real concern that bowel protection comes with a high cost of tumor growth promotion. Furthermore, glutamine concentration is inversely related to tumor growth [15]. Study of Ehrlich carcinoma revealed glutamine concentration has been lower in ascites than in plasma, illustrating the avidity of tumor cells to glutamine [16]. Additional evidence of the tumor's preference to glutamine is an increase in glutamine synthetase and a decrease in glutaminase in kidney-tumor-bearing mice. This ratio is reversed in the late tumor stages when the tumor cells are primarily consuming all glutamine from the host [16]. Cancer cells compete for nitrogen with the host and act as a nitrogen trap. This causes a negative nitrogen balance in the host, which is characterized by weight loss. Indeed, glutamine is the main nitrogen source for tumor cells [17]. Consequently, any compounds which antagonize glutamine are being tested for antitumor activity [17].

Physiologically, the plasma glutamine level is between 0.6 and 0.9 mM [18]. Intracellular glutamine concentration (4–8 mM) is higher than that of plasma due to the active transport system, which allows for a net accumulation of glutamine [19]. Our present experiments attempted to replicate *in vitro* conditions and supraphysiologic states at multiple concentrations of glutamine from 0 (control) to 0.4 (concentration in most commercial culture media), 10, and 20 mM. With regard to radiation technique, the single dose of this experiment differs from

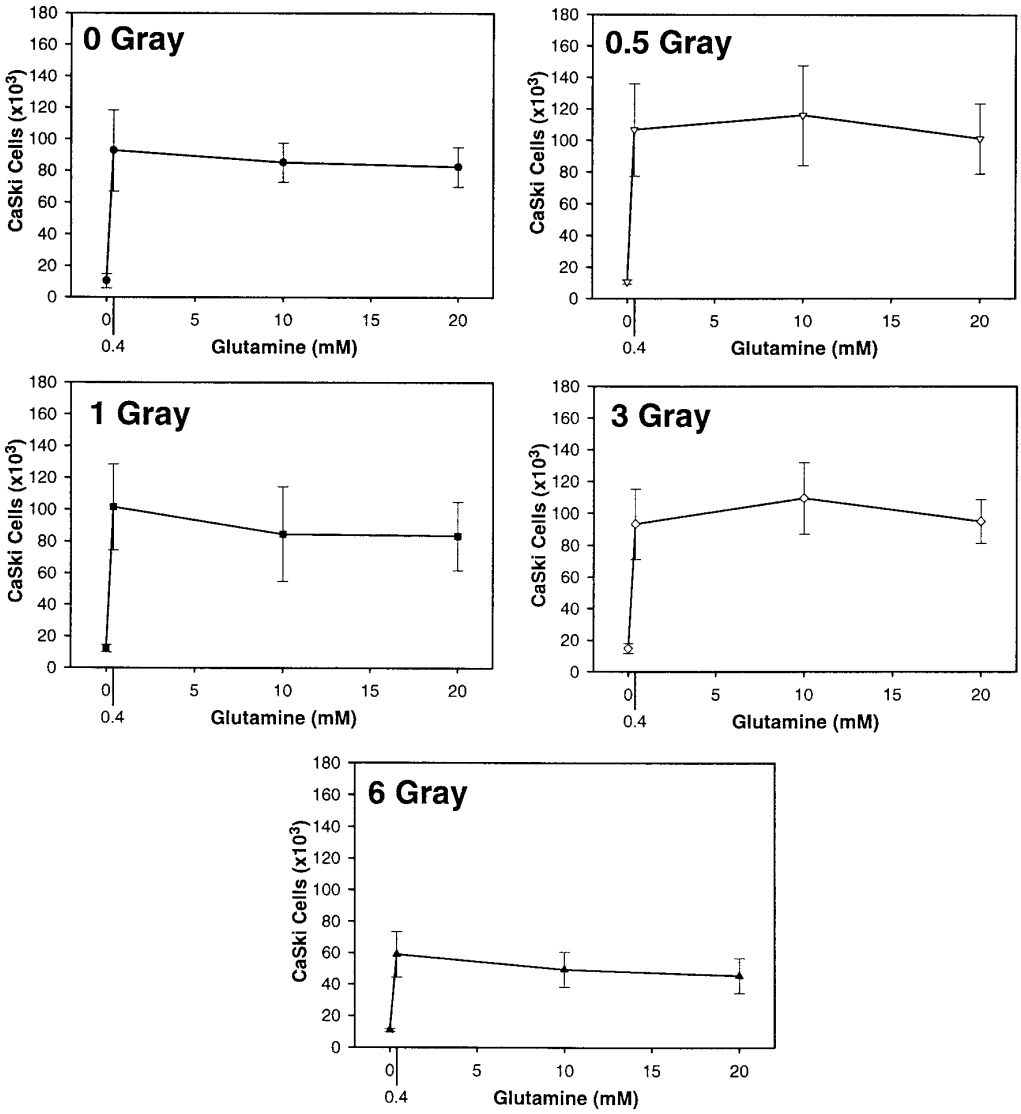

FIG. 2. CaSki cell counts at day 8 ($\times 10^3$). The cells were incubated with varying concentrations of glutamine and irradiated at four different radiation doses (0.5, 1, 3, and 6 Gy) and one control (0 Gy). Cell counts values are mean ± standard deviation of six replicates.

the usual treatment with dose fractionation that lasted for 4–5 weeks. This single-dose radiation was chosen to maximally demonstrate tumoral capabilities for glutamine consumption. Rather than giving fractionated radiation to generate survival curves, smaller total doses were given to allow the cell to grow in varying concentrations of glutamine. Furthermore, it was technically difficult to replicate clinical conditions by maintaining cell viability *in vitro* for 4–5 weeks. Heeneman *et al.* have shown that glutamine becomes unstable in medium after 48 h [20]. Consequently, fresh medium was added every day. Additionally, this practice eliminated the possibility of byproducts of glutamine (glutamate and ammonia) that suppress growth artificially.

Prior to our study, there was real concern about a linear correlation between the amount of glutamine supplementation with tumor growth or the tumor's resistance against radiotherapy. Winters [13] reported growth-enhancing and radioprotective properties of glutamine in irradiated noncancerous ovarian cells. Our results demonstrate that cervical cancer cell lines require a minimal concentration of glutamine of 0.4 mM *in vitro* for adequate growth. However, subsequent increases in glutamine of up to 20 mM did not affect the cell growth or morphology. Furthermore, glutamine does not appear to protect cell growth from radiation injuries. With 6 Gy of radiation, marked growth suppression was seen in both cancer cell lines regardless of the dose of glutamine concentration. CaSki cells showed some variability compared with HeLa cells with respect to their response to radiation levels. Since both HeLa and CaSki were grown and studied in exactly the same experimental conditions, the variability may suggest differences in the

nature of the cell lines. We are currently conducting a similar experiment in different cell lines to confirm this observation.

Our study confirms the recommendation of other studies [5–9, 16] to evaluate glutamine as a bowel protector in irradiated patients. Although glutamine has been found to be radioprotective to normal ovarian cell lines [13] and intestinal cells [5, 6, 8], we do not find a similar pattern in squamous cancer cell lines *in vitro.* The possible protective mechanisms of glutamine to noncancerous cells are probably related to its role as a primary fuel for enterocytes and a precursor for free-radical scavengers such as glutathione [13, 18]. In squamous cancer cell lines, we found that the possible mechanisms noted above only work up to 4 mM glutamine concentration. We speculate that the active transport in cancer cell lines may not be as effective in normal cells. Further study to explain the biologic differences in normal and cancerous cell lines could be achieved by measuring intracellular glutamine levels in these two different cells. At present, a study using glutamine as a gut protector in patients with gynecologic cancer should be pursued. We are currently coordinating a study in gynecologic oncology patients who are receiving pelvic/abdominal radiation. These patients will be randomized to receive oral glutamine supplementation versus placebo. We hypothesize that patients who receive glutamine supplementation may have less acute and chronic gastrointestinal toxicities from the radiotherapy.

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