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GLUTAMINE PROTECTS CHINESE HAMSTER OVARY CELLS FROM RADIATION KILLING

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Summary

Chinese Hamster Ovary (CHO) cells were propagated in vitro and exposed to varying doses of ionizing radiation. The surviving fraction of cells was determined, being found to be a function of the radiation dose. The cell survival curves obtained as a function of radiation dose were modified by the inclusion of varying doses of glutamine in the medium with glutamine demonstrating a radioprotective effect. The radioprotectant effect of glutamine for CHO cells was more pronounced at higher radiation doses. Glutamine has been categorized as a non-essential amino acid in that it can be synthesized in some tissues; however, a number of cell lines require glutamine to survive and grow in vitro and supplementation of glutamine has been found to ameliorate the stress of surgery or irradiation to the gastrointestinal tract. It may be appropriate to consider glutamine as a conditionally essential amino acid.

Key Words: glutamine, ovary, radiation

Ionizing radiation finds a practical application in medicine in the area of oncology since malignant neoplasms are generally sensitive to it. The eradication of human cancers by irradiation is limited by side effects and complications induced in nearby normal tissues. Growing tissues such as the epithelium that lines the gastrointestinal tract are frequently susceptible to such problems (1). Measures which would protect normal tissues while permitting destruction of a malignant neoplasm would have an apparent advantage. Previous studies have established that glutamine confers some radioprotectant effect against the gastrointestinal tract toxicity of irradiation (2). The present study was undertaken to determine whether or not a radioprotectant effect of glutamine could be demonstrated in a simpler in vitro system, or whether this effect of glutamine would be restricted to a more complex structure such as the gut.

Materials and Methods

Maintenance and manipulation of cells

CHO cells, which are an established tumor cell line, were propagated in Hams F-12 culture medium supplemented with 10% fetal calf serum (FCS). After trypsinization, cells were collected and centrifuged at 1000xg for 5 minutes. The resulting cell pellets

were resuspended in fresh medium and counted on a hemacytometer. Enough cells were plated into small petri dishes to obtain approximately 100 colonies following irradiation and incubation. The plated cells were covered with 5 mL of fresh medium containing the desired concentration of glutamine given in the form of the dipeptide alanylglutamine. Each group of cells irradiated at the same dose and receiving the same glutamine concentration were plated in triplicate. The cells were incubated for 3 hours and irradiated at various doses using 9 MeV electrons. The cells were then incubated for 7-10 days.

Staining and counting of colonies

Following the 7-10 day incubation period, the resulting cell colonies were stained with methylene blue and counted. The expired cell media was aspirated off and enough methylene blue solution was added to cover the bottom of the petri dish. After a 15 minute time period, the methylene blue solution was poured off and the petri dishes were rinsed with distilled water to completely remove all dye solution. The petri dishes were then allowed to air dry and the cell colonies were counted.

Construction of cell survival curves and calculation of dose modifying factor (DMF)

Cell survival curves were constructed by plotting the surviving fraction (number of colonies counted divided by the number of cells plated times the plating efficiency) on a logarithmic scale versus radiation dose in Gray plotted on a linear scale. Dose modifying factors, a measure of the effectiveness of a radioprotector, were calculated by dividing the radiation dose required to give a 0.1 surviving fraction for cells incubated in higher concentrations of glutamine (experimental group) by the radiation dose required to give a surviving fraction of 0.1 for cells incubated in 1 mM glutamine (control group). A DMF greater than 1.0 indicates that glutamine was exerting a radioprotective effect.

Results

In the first set of experiments performed, cell survival curves were generated for cells incubated in various glutamine concentrations. Figure 1 shows cell survival curves obtained for 1 mM, 5 mM, 10 mM, and 20 mM glutamine contained in the cell culture medium. Table 1 gives the values for the surviving fractions at various radiation dosages for different medium glutamine concentrations. At each point on the cell survival curves, the surviving fractions are higher in the curves for the higher medium glutamine concentration. This indicates that glutamine is exerting a radioprotective effect and that this radioprotective effect is increased with an increase in the medium glutamine concentration.

A dose modifying factor (DMF) was obtained for each of the cell survival curves shown in Figure 1. The values for the DMF for each of the survival curves are given in Figure 1. Figure 2 is a plot of DMF versus glutamine concentration in the cell culture medium. There is an increase in DMF with an increase in glutamine concentration that appears to be linear. This shows that a higher dose of radiation is necessary to obtain the same surviving fraction in cells incubated in a higher concentration of glutamine than in cells incubated in a lower concentration of glutamine.

Another set of experiments was performed by varying the glutamine concentration in the media of cells irradiated at the same dose of radiation. Table 2 shows the surviving fraction as a function of glutamine concentration for cells irradiated at 2 Gy

FIG. 1

Survival curves for CHO cells incubated in different concentrations of glutamine, showing dose modifying factors for each glutamine concentration's survival curve.

TABLE I

Radiation Dose (Gy)	1 mM Gln	5 mM Gln	10 mM Gln	20 mM Gln
2	0.746 ± 0.00531	0.850 ± 0.0523	0.922 ± 0.0617	0.970 ± 0.00990
4	0.278 ± 0.0168	0.398 ± 0.00200	0.450 ± 0.0149	0.500 ± 0.00195
6	0.0680 ± 0.00343	0.0999 ± 0.0173	0.133 ± 0.0117	0.184 ± 0.000
8	0.0200 ± 0.00436	0.0340 ± 0.00210	0.0481 ± 0.00515	0.0520 ± 0.0103
10	0.00380 ± 0.0000990	0.00769 ± 0.000615	0.0128 ± 0.00280	0.0149 ± 0.0000950

Surviving fractions of CHO cells incubated with varying concentrations of glutamine that were irradiated at various doses of radiation. Surviving fraction values are mean ± standard deviation of at least three replicates.

FIG. 2

Dose modifying factors of cell survival curves of irradiated CHO cells versus the glutamine concentration in the CHO cell culture medium.

TABLE II

Gln concentration (mM)	Surviving Fraction, 2 Gy	Surviving Fraction, 10 Gy
0.0	0.729 $\pm$ 0.0811	
0.5	0.819 $\pm$.0218	
1.0	0.835 $\pm$ 0.103	0.00625 $\pm$ 0.000538
2.0	0.893 $\pm$ 0.1301	0.00682 $\pm$ 0.000
3.0		0.00736 $\pm$ 0.000896
4.0		0.00852 $\pm$ 0.002105
5.0		0.00965 $\pm$ 0.001442

Surviving fractions of CHO cells irradiated at either 2 Gy or 10 Gy containing various concentrations of glutamine in the cell medium. Each surviving fraction value is the mean $\pm$ standard deviation of at least three replicates.

and 10 Gy. There is an increase in surviving fraction with increase of glutamine concentration at both doses of radiation. This increase in surviving fraction with

increase in glutamine concentration is greater in the cells irradiated at 10 Gy. This may indicate that glutamine is exerting a greater radioprotective effect at higher doses of radiation. Figure 3 shows a plot of surviving fraction versus glutamine concentration for cells irradiated at 10 Gy. A linear least squares fit of these data gives a correlation coefficient (R^2) value of 0.967, compatible with a linear relationship of surviving fraction to glutamine concentration.

FIG. 3

A plot of surviving fraction of CHO cells irradiated at 10 Gy versus the glutamine concentration contained in the cell culture medium.

Discussion

Since glutamine can be synthesized in most animal cells, it has traditionally been regarded as a non-essential amino acid. In some instances, however, the need for glutamine apparently outweighs the body's ability to produce it. An increased need for glutamine occurs during catabolic stresses such as those found following infection (3,4), major surgery (5), and, of particular importance to the present study, following radiation therapy of cancer patients (2).

During radiation treatment, and especially during irradiation of the abdomen, the gut becomes a system of major importance. Many of the side effects of irradiation are gastrointestinal in nature, and include nausea, vomiting, and diarrhea. These side effects can become severe and may be a contributing factor to complications seen following radiation treatment. During the catabolic stress induced by irradiation, the gut's need for glutamine apparently surpasses the body's ability to supply it, and increased intake of glutamine becomes essential. A previous study from our laboratory shows that the plasma level of glutamine is significantly decreased in mice following whole-abdomen irradiation (6). Other studies have shown that glutamine supplementation following irradiation serves to alleviate some of the gastrointestinal-associated side effects (2,7), raise the plasma level of glutamine (7), improve gut morphology (5,7), and increase survival following whole-abdomen irradiation (8).

Glutamine's role for the gut following irradiation has not been completely elucidated.

Since glutamine can be metabolized to many products, there are several possibilities for how glutamine protects the gut from radiation damage. These possibilities include glutamine acting as an energy substrate for the gut (9), glutamine preventing bacterial translocation from the gut (10,11), or glutamine acting as a direct radioprotector and either serving to prevent radiation damage or serving to enhance the repair process following irradiation.

Our results in the present study show that glutamine is acting as a radioprotector in a simple *in vitro* system, as opposed to a complicated *in vivo* system such as the gut. Our results do not support the hypothesis that glutamine protects the gut from radiation damage solely by preventing bacterial translocation from the gut, since glutamine is shown to be a radioprotector in a simple *in vitro* system where the possibility of a phenomenon such as bacterial translocation does not exist. While glutamine may prevent bacterial translocation from the gut following irradiation, our results indicate that glutamine has a more direct effect in protecting mammalian cells. Protection of the cells in the gut may serve to prevent bacterial translocation from the gut.

It is possible that glutamine's role as an energy substrate facilitates its usefulness as a radioprotector. Repair of damage caused by radiation exposure requires energy, which could be provided by the supplemental glutamine included in the growth medium. Glutaminase converts glutamine to glutamate, which can be converted to α -ketoglutarate, which can enter the Krebs cycle with a large net output of ATP. Many cell lines prefer glutamine as an energy substrate, and glutamine is essential for their growth and survival in tissue culture. It should be noted that supplementation of glutamine from 1 mM to 20 mM did not increase the growth rate of non-irradiated cells. The plating efficiency (survival of non-irradiated cells) did not change with increasing glutamine concentration, except for a slight decrease in plating efficiency with increasing glutamine concentration observed when glutamine was given in the form of L-glutamine. This is probably due to the instability of glutamine in aqueous solution and to the toxicity of ammonia obtained from the breakdown of glutamine. This decrease in plating efficiency with increase in glutamine concentration was not observed when glutamine was given in the form of the dipeptide alanyl-glutamine.

Since the clonogenic assay used in the present study depends upon the reproductive viability of the irradiated cells, it is possible that glutamine is involved in the DNA repair process following radiation exposure. Exposure to ionizing radiation can cause the formation of hydroxyl radicals which can react with a cell's DNA to form lesions which must be repaired in order to preserve the reproductive viability of the cell. This repair process requires a supply of free purine and pyrimidine nucleotides. Glutamine's amide nitrogen can be used to form purine and pyrimidine bases (12). Glutamine supplementation may increase the number of free nucleotides available, which might speed the DNA repair process.

A final possibility for glutamine's observed radioprotective effect is glutamine acting as a direct radioprotector and preventing damage by free radicals formed during irradiation. This may occur by some as yet unknown process, or glutamine may be a precursor for a known radioprotector. Several radioprotectors have been studied, including cysteine and cysteamine. These radioprotectors exert their radioprotective effect by scavenging free radicals that form following exposure to ionizing radiation by reacting with free radicals at the sulfhydryl group of the radioprotector. One of the major radioprotectors found in the body is the tripeptide glutathione (γ -

glutamylcysteinylglycine), which consists of the amino acids glutamic acid, cysteine, and glycine. Glutathione is usually present in high levels in the body and, in addition to serving several protective functions, exerts a radioprotective effect by scavenging free radicals in the same manner as cysteine and cysteamine. Glutathione has been considered by some to be a reservoir of cysteine, offering the radioprotective effects of cysteine in a form preferred by the body over the form of cysteine (13,14). Glutamine is converted to glutamic acid and ammonia by the enzyme glutaminase. If availability of glutamic acid was limiting in the formation of glutathione, glutamine supplementation could increase the concentration of glutamate and thus increase the concentration of the known radioprotector glutathione.

Since glutamine is shown to be a radioprotector, glutamine supplementation following radiation therapy might be beneficial for the cancer patient receiving radiation therapy. A potential disadvantage of glutamine supplementation for cancer patients is the possibility of tumors using glutamine as an energy substrate and glutamine supplementation actually spurring the growth of a tumor. Previous studies have shown that glutamine supplementation following irradiation of mice prevents radiation enteropathy (15) and that glutamine-enriched total parenteral nutrition of tumor-bearing mice does not increase tumor size, tumor DNA content, or tumor glutaminase activity (16). The results of these studies and the present study indicate that glutamine supplementation following radiation therapy might exert a radioprotective effect to healthy tissue without spurring tumor growth.

In conclusion, the results presented indicate that glutamine acts as a radioprotector in a simple *in vitro* system. These results support the idea that glutamine protects body systems such as the gut more directly as a radioprotector as opposed to a more indirect route, such as preventing bacterial translocation from the gut. This simplifies somewhat the questions surrounding glutamine's role in radiation stress.

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