

Elimination of Hypoxic Protection by 5-Thio-D-glucose in Multicell Spheroids¹

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ABSTRACT

The effect of 5-thio-D-glucose on oxic and hypoxic V79-171 Chinese hamster cells was studied *in vitro* with single cells and multicell spheroids. At concentrations that were not toxic to oxic cells, this compound killed hypoxic cells with a D_0 of 1 hr with a 5 mM concentration and more rapidly at 10 mM beginning 2 hr after incubation at 37°. 5-Thio-D-glucose also sensitized hypoxic cells to radiation and protected oxic cells from radiation damage. Multicell spheroids irradiated after incubation with the compound demonstrated increased radiosensitivity, although the relative contribution of cytotoxicity and hypoxic cell sensitization could not be evaluated. Spheroid reoxygenation by decreased cell respiration was determined not to be a contributing factor, suggesting that the spheroid-sensitizing effect was due to drug effects on hypoxic cells. The dramatic increase in multicell spheroid radiosensitivity that resulted from treatment with 5-thio-D-glucose suggests that this compound may be used to increase the effectiveness of radiotherapy by eliminating hypoxic protection.

INTRODUCTION

During the last several years, numerous organic nitro compounds have been reported to be able to kill hypoxic cells and also to sensitize hypoxic cells to radiation (1, 6, 7, 9, 14). Some of these compounds are being tested for clinical use in conjunction with radiotherapy for the elimination of hypoxic protection (16).

The most prominent metabolic difference between aerobic and hypoxic cells is their glucose metabolism. Aerobic or oxic cells are relatively resistant to depletion of glucose supply or to inhibition of glycolysis by virtue of their ability to produce energy by respiration with endogenous substrates such as fatty acids. On the other hand hypoxic cells obtain their energy entirely by anaerobic glycolysis; thus, they are vulnerable to diminished glucose supply or to inhibition of glycolysis. 5-SH-Glc³ is the nearest chemical analog and a potent inhibitor of active transport of D-glucose and glycolysis (2, 17). We have previously tested the effect of 5-SH-Glc on mouse mastocytoma cells and found it to be preferentially cytotoxic and radiosensitizing

to acutely hypoxic cells (10, 11). Furthermore, 5-SH-Glc selectively potentiates hyperthermic killing of hypoxic tumor cells (8). There is a possibility, however, that the response of chronically hypoxic tumor cells to 5-SH-Glc might be different from that of acutely hypoxic cells. In this study we have investigated the effect of 5-SH-Glc on acutely hypoxic single cells and chronically hypoxic cells in multicell spheroids of Chinese hamster cells.

MATERIALS AND METHODS

Cytotoxicity of 5-SH-Glc on Hypoxic Cells

Chinese hamster V79-171 cells were obtained from Dr. R. Durand of Johns Hopkins University. Stock cells were grown at 37° in RPMI Medium 1640 containing 100 μ g streptomycin per ml and 100 units of penicillin per ml and were supplemented with 10% FCS (Associated Biomedic Systems, Inc., Buffalo, N. Y.) under an atmosphere of 95% air and 5% CO₂. (Hereafter this condition will be referred to as standard culture conditions.) Exponentially growing monolayer stock cells were harvested by treatment with 0.025% trypsin, and appropriate numbers of cells were pipeted into glass T-flasks. After overnight incubation under standard culture conditions, the media were replaced with fresh media alone, or media containing various amounts of 5-SH-Glc (M. W. 196.3; Pfanstiehl Laboratories, Inc., Waukegan, Ill). For induction of hypoxia the flasks were closed with solid-rubber stoppers that contained two 20-gauge needles. The flasks were gassed through one of the needles with a mixture of 95% N₂ and 5% CO₂ humidified with 37° distilled water. The flow rate was about 0.5 liter/min/flask. The gas contained oxygen less than 100 ppm, according to the manufacturer's specifications. After incubation at 37° for 1 to 8 hr with gentle rocking, the cells were gently rinsed 3 times with warm RPMI Medium 1640 and the cells were cultured for 7 to 8 days under standard culture conditions. The clones were stained with crystal violet and the surviving fraction calculated.

Effect of 5-SH-Glc on Cell Radiosensitivity

Cells were incubated with or without 5 mM 5-SH-Glc under aerobic and hypoxic conditions for 4 hr at 37° with gentle rocking as described previously. The needles were removed but maintained a positive gas pressure in the flasks. The cells were irradiated with various doses of X-rays. The radiation factors were 220 kV X-rays, 15 ma, added filtration of 1 mm aluminum and 0.25 mm copper, and a dose rate of 270 rads/min. After the irradiation the cells were washed with fresh media and incubated for 7 to 8 days, and the clones were stained with crystal violet.

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³ The abbreviations used are: 5-SH-Glc, 5-thio-D-glucose; RPMI, Roswell Park Memorial Institute; FCS, fetal calf serum.

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Effect on Chronically Hypoxic Cells in Spheroids

Spheroids of V79-171 cells were grown as described by Sutherland and Durand (5, 15). Briefly, single-cell suspensions were prepared from exponentially growing stock cultures, and the cell concentration was adjusted to 2×10^4 cells/ml in RPMI Medium 1640, containing antibiotics and supplemented with 5% FCS. The cells were transferred to spinner flasks and cultured at 37° under 5% CO₂ and 95% air with stirring at 180 rpm in an Incu-cover (Associated Biomedic Systems, Inc., Buffalo, N. Y.). From the fourth day of culture, the medium was changed every day until the spheroids grew to about 1 mm in diameter in 20 to 25 days. For the study of the effect of 5-SH-Glc on chronically hypoxic cells in the spheroid, about 200 spheroids were suspended in 100 ml of RPMI Medium 1640 that contained 5 mM 5-SH-Glc in water-jacketed spinner flasks and incubated for 17 hr at 37° under 5% CO₂-95% air. The control spheroids were treated the same, except the incubation medium was free of 5-SH-Glc. The spheroids were then irradiated with X-rays. The spheroids were continuously stirred under the CO₂-air mixture during the irradiation. It has been reported that the oxygen supply to the internal cells of the spheroids is rather sensitive to the temperature of the media (15). We, therefore, maintained the temperature of spheroids at 37° by circulating 37° water through the water jacket when the flasks were taken out of the incubator for irradiation. The irradiation factors were the same as described previously, except the dose rate was 250 rads/min. After irradiation at graded doses, a portion of the spheroids was removed into plastic culture tubes. They were dissociated to single cells by incubation with 0.25% trypsin solution containing a small amount of DNase for 10 min at 37° with rocking and by repeated pipeting. The enzymatic activity was stopped by RPMI Medium 1640 supplemented with 20% FCS; the number of viable cells was counted by the trypan blue exclusion method, and appropriate numbers of cells were plated in plastic T-culture flasks. After 7 to 8 days of culture under the standard culture conditions, the clones were stained with crystal violet and the surviving fraction was calculated.

Changes in the total number of clonogenic cells per spheroid after treatment with 5-SH-Glc were determined in a separate set of experiments. About 200 spheroids, 0.7 mm in diameter, were suspended in 100 ml of fresh RPMI Medium 1640 containing 0 or 5 mM 5-SH-Glc. After a 17-hr incubation period, 10 spheroids from each suspension were transferred into test tubes. The spheroids were dissociated enzymatically and mechanically as described above, diluted with media containing 20% FCS, and plated. Clones were counted 8 days later.

Effect of 5-SH-Glc on the O₂ Consumption Rate of Cells

The effect of 5-SH-Glc on cellular oxygen consumption was studied with P815-X2 mastocytoma cells of DBA/2J mice and Chinese hamster V79 cells.

P815-X2 Mastocytoma Cells. Stock cells growing exponentially in RPMI Medium 1640 supplemented with 10% FCS were harvested and incubated with 0 or 10 mM 5-SH-Glc in RPMI medium 1640 for 1, 4, and 7 hr. At the end of the incubation period, a known number of cells were placed

in a 10-ml gas-tight syringe, immersed into a 37° water bath, and incubated. The amount of O₂ in 0.5-ml aliquots of cell suspension was determined at approximately 12-min intervals for 1 hr by means of a modified Hersch cell (Coulometer). A detailed description of the apparatus and techniques has been published elsewhere (3).

V79 Cells. Cells growing exponentially in glass T-flasks were incubated with 0 or 10 mM 5-SH-Glc in RPMI Medium 1640 for 4 hr. The cells were then harvested with 0.025% trypsin. A known number of cells was resuspended in RPMI Medium 1640 that contained 0 or 10 mM 5-SH-Glc, and placed in a 10-ml gas-tight syringe. The O₂ consumption rate of the cells was determined from the change in O₂ content in the cell suspension as described previously.

RESULTS

Chart 1 shows that 5-SH-Glc was extremely cytotoxic to hypoxic V79 cells at concentrations nontoxic to aerobic cells. The cells started to die about 2 hr after the initiation of incubation with 5 or 10 mM 5-SH-Glc under hypoxic conditions. The surviving fraction decreased exponentially as a function of incubation time. Less than 10 and 1% of the hypoxic cells survived a 4-hr incubation with 5 and 10 mM 5-SH-Glc, respectively. On the other hand incubation with 5-SH-Glc at a concentration of 10 mM for 8 hr had no significant effect on the survival of aerobic V79 cells.

The effect of 5-SH-Glc on the radiosensitivity of aerobic

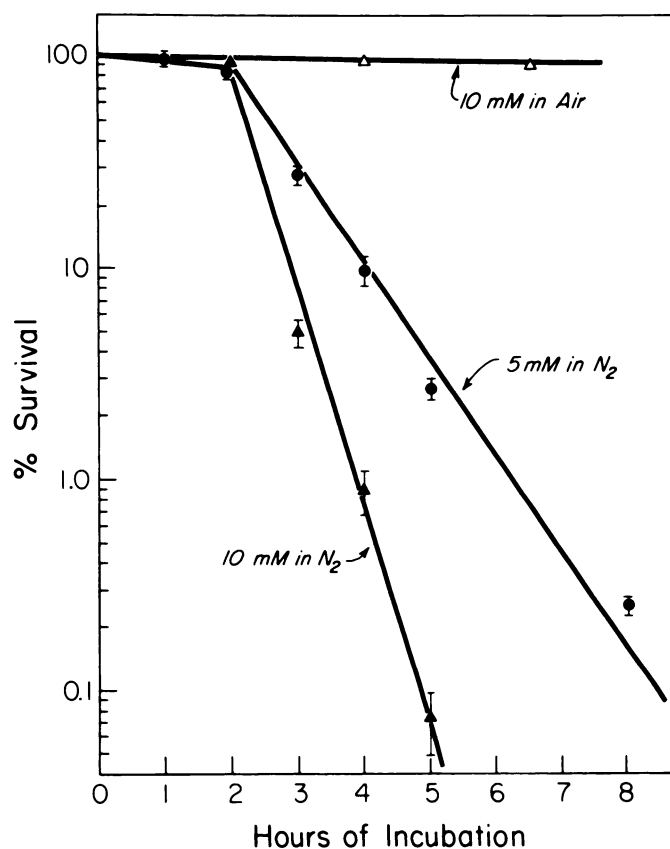


Chart 1. Changes in percentage of survival of Chinese hamster V79-171 cells as a function of incubation time at 37° under aerobic and hypoxic conditions with 5-SH-Glc. Points, averages of 5 to 7 experiments; bars, S.E.

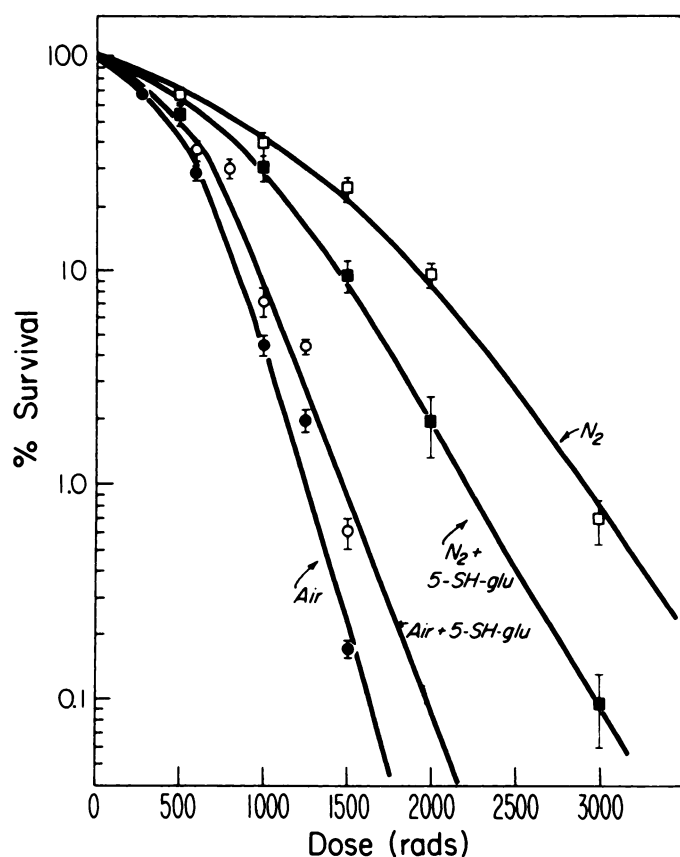


Chart 2. Effects of 5-SH-Glc (5-SH-glu) on radiosensitivity of monolayer Chinese hamster V79-171 cells. The cells were incubated with 5 mM 5-SH-Glc for 4 hr at 37° under aerobic and hypoxic conditions and irradiated. Points, averages of 5 to 7 experiments; bars, S.E.

and hypoxic V79 cells is shown in Chart 2. Each of the survival curves has been normalized, so that 100% survival is based on the clonogenicity of each group at the time of irradiation. Under aerobic conditions V79 cells had a D_{10} and N of 150 rads and about 20, respectively. The corresponding values under hypoxic conditions were 400 rads and about 20. Treatment with 5-SH-Glc for 4 hr prior to irradiation slightly decreased the radiosensitivity of aerobic cells, and significantly increased the radiosensitivity of hypoxic cells; the D_{10} increased from 150 to 190 rads under aerobic conditions and decreased from 400 to 270 rads under hypoxic conditions. Thus, 5-SH-Glc reduced the oxygen enhancement ratio from 2.7 to 1.4.

Table 1 shows the effect of 5-SH-Glc on the total number of clonogenic cells per multicell spheroid. The number of clonogenic cells is 5.6×10^4 and 4.2×10^4 cells in the control and treated spheroids, respectively. This reflects a 25% reduction in clonogenic cells in the spheroids that resulted from incubation of the spheroids with 5 mM 5-SH-Glc for 17 hr.

Chart 3 shows the radiation response curves of the cells from control spheroids of 1-mm diameter was multiphasic. The radioresistant final portion is believed to represent the radioresponse of noncycling hypoxic cells (12). A back-extrapolation of this radioresistant terminal portion of the survival curve to the 0-dose axis suggested that 15 to 20% of the clonogenic cells in the spheroids were radiobiologically hypoxic.

Treatment of spheroids with 5 mM 5-SH-Glc for 17 hr prior to irradiation significantly changed the shape of the survival curve. The increase in the slope of the terminal portion indicated that the remaining cells in the spheroids treated with 5-SH-Glc were more radiosensitive as compared with untreated hypoxic cells in the control spheroids.

The effect of 5-SH-Glc on oxygen consumption is shown in Table 2. These data indicate that 5-SH-Glc causes a significant increase in respiration rate of both mastocytoma cells and V79 cells. Maximum O_2 consumption, which was about 70 to 90% above control, occurred during the 1- to 4-hr incubation periods. Increased O_2 consumption is probably due to an increase in oxidative respiration of cells that used endogenous substrates such as fatty acids to compensate for the drug-induced decrease in energy production through glycolysis.

Table 1
Total number of clonogenic cells of V79 cells in spheroids treated with 0 mM or 5 mM 5-SH-Glc for 17 hr
The diameter of the spheroids was 700 μ m.

Treatment	Total clonogenic cells/spheroid ($\times 10^4$)
0 mM	5.6 ± 0.30^a
5 mM	4.2 ± 0.30^b

^a Mean \pm S.E. of 6 measurements.

^b Significantly different from 0 mM groups ($p < 0.05$).

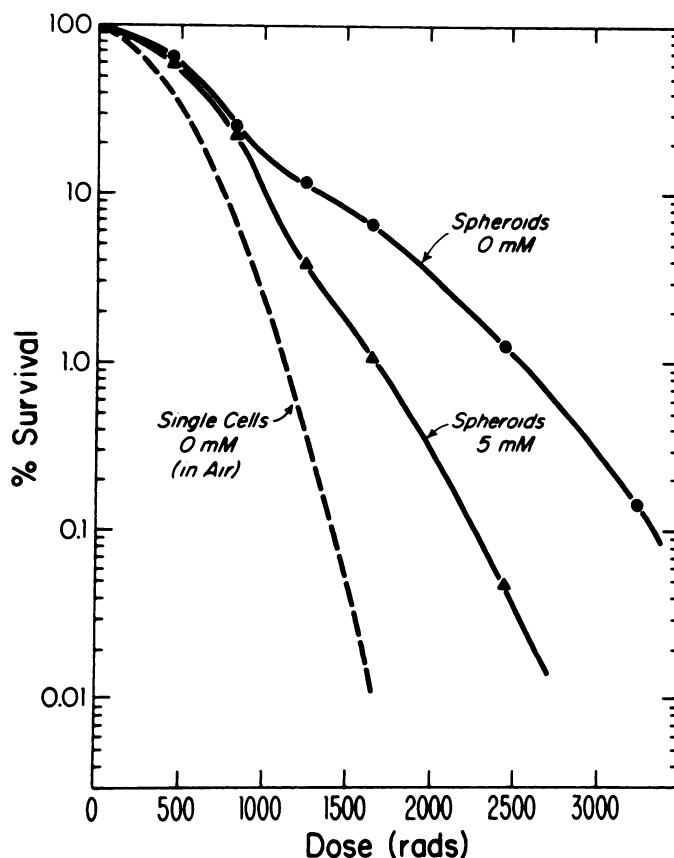


Chart 3. Radiation survival curves of Chinese hamster V79-171 cells from control spheroids and spheroids incubated with 5 mM 5-SH-Glc for 17 hr. Averages of 5 experiments are shown. ---, is the survival curve of aerobic monolayer cells.

Table 2
Changes in cellular oxygen consumption rate following incubation with 10 mM 5-SH-Glc for 1 to 7 hr

Cell type	Treatment	Oxygen consumption rate ($\mu\text{M O}_2/10^6$ cells/min)	% of control
V79	Control	0.1831 \pm 0.0167 ^a	100
	5-SH-Glc, 10 mM, 4 hr ^b	0.3318 \pm 0.0821	170 ^c
P815-X2	Control	0.1482 \pm 0.0076	100
	5-SH-Glc, 10 mM, 1 hr	0.2654 \pm 0.0287	179 ^c
	5-SH-Glc, 10 mM, 4 hr	0.2766 \pm 0.0276	187 ^c
	5-SH-Glc, 10 mM, 7 hr	0.1915 \pm 0.0156	129

^a Mean \pm S.E.

^b Hr of treatment before O₂ consumption rate measurements.

^c Significantly different from controls ($p < 0.01$).

DISCUSSION

The results of the present study with monolayer Chinese hamster V79-171 cells confirm our previous observation with mastocytoma P815-X2 cells in suspension culture (10, 11) that 5-SH-Glc specifically kills hypoxic cells, sensitizes hypoxic cells to radiation, and protects aerobic cells from radiation damage. Our results with multicell spheroid further demonstrate that 5-SH-Glc increases the radiosensitivity of a mixed cell population in a tumor-like environment.

Chart 1 indicates that the surviving fraction began decreasing 2 hr after the initiation of incubation with 5 or 10 mM 5-SH-Glc under hypoxic conditions. We reported previously that the lag period of mastocytoma P815-X2 cells was also about 2 hr (10, 11). The rate of V79 cell death after the lag period, however, appears to be different from that of mastocytoma cells. The D_{10} (time) of V79 cells at 5 mM 5-SH-Glc was about 1 hr, whereas it was about 2 hr with the mastocytoma cells (11). It is not clear whether the difference in survival kinetics between V79 cells and mastocytoma cells results from an inherent difference in drug sensitivity or from a difference in experimental conditions. The V79 cells were in monolayer form, covered with about 0.5-cm-deep media in T-flasks, whereas the mastocytoma cells were in suspension in about 1-cm-deep media. It is possible that the dissipation of oxygen from the 1-cm-deep suspension of mastocytoma cells was not as effective as that from the 0.5-cm-deep V79 cell culture, resulting in slower death of the mastocytoma cells.

Hypoxic V79 cells that survived treatment with 5-SH-Glc are more radiosensitive than the untreated hypoxic cells. It is possible that 5-SH-Glc and radiation synergistically affect the cells. The mechanism of the radioprotective effect of 5-SH-Glc on oxic cells is not clear, but it may be that cells are partially synchronized at a radioresistant phase in the cell proliferation cycle by 5-SH-Glc, or free sulfhydryl groups are produced due to a metabolic breakdown by 5-SH-Glc.

Although induction of hypoxia by gassing with nitrogen is abrupt, hypoxia in tumors develops gradually as the tumors outgrow their vascular supply. A response of such chronically and perhaps noncycling hypoxic cells in the

tumors to various chemotherapeutic agents and radiation may be different from that of acutely hypoxic cells. The *in vitro* multicell spheroids have been reported to be similar to *in vivo* tumors in that a portion of the cell population becomes chronically hypoxic with age (5, 15). The reduction in total clonogenic cells/spheroid after treatment with 5-SH-Glc (Table 1) is attributed to the preferential cytotoxicity of this drug to hypoxic cells. While cytotoxicity of 5-SH-Glc to oxic cells has been reported for 7.5 mM concentrations for 3 days (13) or 10 days (11), exposure of cells for 8 hr to concentrations up to 25 mM had no significant cytotoxic effect on oxic cells (11). Our previous histopathological studies support the contention that 5-SH-Glc kills hypoxic cells in spheroids (12). Staining with hematoxylin and eosin, periodic acid-Schiff, and Masson trichrome clearly demonstrated that treatment of spheroids with 5-SH-Glc preferentially kills the cells of the inner part of the viable rim of spheroids, which are most likely hypoxic cells.

Based on single-cell cytotoxicity data (Chart 1), a 17-hr incubation of spheroids with 5-SH-Glc would be expected to eliminate virtually all hypoxic spheroid cells. Data in Chart 3 suggest either the presence of a hypoxic population with increased radiosensitivity or an oxic population with increased radioresistance. Increased radioresistance of oxic cells produced by 17 hr 5-SH-Glc treatment of spheroids may be greater in magnitude than that found for single cells treated for 4 hr, since we have noted that the degree of radioresistance increases with length of incubation (11). If, however, the terminal portion of the survival curve for treated spheroids represents the radiation response of hypoxic cells, the increased sensitivity of spheroids as reflected in the steepness of the slope can be attributed either to hypoxic cell sensitization or to reoxygenation of hypoxic cells. Durand *et al.* (4) reported that the sensitization of hypoxic cells in spheroids by some hypoxic cell sensitizers results not from a direct sensitization but from a reoxygenation due to reduced oxygen consumption by the outer cells in the spheroids. The effect of 5-SH-Glc on hypoxic cells in the spheroids is not due to a reoxygenation of hypoxic cells, since 5-SH-Glc increases rather than decreases the oxygen consumption, as shown in Table 2.

In summary increased radiation sensitization of multicell spheroids treated with 5-SH-Glc has been conclusively demonstrated. Although the relative contribution of hypoxic cell cytotoxicity and cell radiosensitization cannot be quantitated, both of these phenomena are clearly seen for single-cell cultures. It has been further shown that spheroid sensitization is not due to reoxygenation by drug-induced respiratory inhibition. The unique ability of 5-SH-Glc to kill and radiosensitize hypoxic cells preferentially and its low systemic toxicity to animals (18) warrant the testing of the potential of this compound to eliminate hypoxic protection during radiotherapy of *in vivo* tumors.

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