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The effect of glycolysis inhibitors on the radiation response of CHO-K1 cells

C.B. Seymour[1] and C. Mothersill[1,2]

[1] Saint Luke's Hospital, Rathgar, Dublin 6, Ireland
[2] Dublin Institute of Technology, Kevin Street, Dublin 8, Ireland

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Summary. Exposure of CHO-K1 cells to three different inhibitors of glycolysis, prior to treatment with a single dose of ionising radiation, reduced their survival. The effects were concentration-dependent but occurred under all conditions where cells were exposed to the inhibitors prior to irradiation. The results are similar to those obtained by this group when glycolysis was altered using analogues of D-glucose or by blocking the pyruvate→lactate reaction using added lactate or oxamate. They support data from other workers suggesting a role for energy metabolism in the final expression of radiation damage.

Introduction

The effect of energy metabolism on the survival and recovery of cells exposed to ionising radiation remains to be clarified. Several reports have been published showing that substances which affect energy metabolism also reduce radiation survival (Alper 1979; Kiefer 1971; Nishizawa et al. 1979; Reinhard and Pohlit 1976; Seymour and Mothersill 1981a; Seymour et al. 1985) but because many of these substances affect other cellular functions it is difficult to pinpoint their role in the development/prevention of cellular radiation damage. In addition, since many mammalian cells have several mechanisms for bypassing metabolic blocks and for producing ATP by secondary pathways (Ashby et al. 1969; Lehninger 1983), interpretation of results must be cautious.

Of particular interest to radiobiologists are the questions of whether ATP or energy metabolism is a "limiting substance" for the repair of radiation damage (Alper 1979) and whether the size of the shoulder of the mammalian survival curve can be equated with a concentration of repair factor available to the cell (Alper 1979; Orr et al. 1966; Powers 1962; Seymour et al. 1983).

As one approach to this problem we have been studying the radiation response of CHO-K1 cells following treatment with a variety of substances

known to affect the glycolytic pathway. The cell line used is capable of a highly anaerobic type of metabolism and under the conditions used converted over 92% of available glucose to lactate (Seymour and Mothersill 1981 b; Seymour 1983). This makes the line highly suitable for studies of glycolytic metabolism. Our previous studies have shown that substances which act as analogues of glucose (2-deoxyglucose, L-glucose) and substances which block or reverse the direction of the LDH reaction (lactate and oxamate) significantly reduce the survival of CHO-K1 cells to single doses of radiation (Seymour and Mothersill 1981 a; Seymour et al. 1984).

We now present evidence that inhibition of glucose breakdown using three different glycolysis inhibitors reduces the survival of irradiated CHO-K1 cells and that the effect is mainly due to a reduction in the survival curve shoulder. The inhibitors chosen were sodium iodoacetate which blocks glycolysis at the glyceraldehyde-3-phosphate dehydrogenase reaction (Racker 1970), sodium arsenate which allows glycolysis to proceed but prevents substrate phosphorylation of ADP→ATP at the 2–3 diphosphoglycerate reaction (Lehninger 1983), thus reducing the level of ATP formed, and sodium fluoride which inhibits the enolase reaction. To exclude the possibility that the cells were using aerobic metabolism to any significant degree two inhibitors of aerobic respiration were tested for their ability to reduce glucose breakdown, cell growth or radiation survival.

Methods

The methods have been described in detail previously (Seymour and Mothersill 1981 a). Briefly, CHO-K1 cells (Flow Laboratories, Scotland) were maintained in Ham's F-12 nutrient mixtures supplemented with 10% foetal calf serum (Gibco Biocult Ltd., Scotland). They were routinely subcultured using Trypsin (0.2%) and Versene (0.02%, 1:1 V/V).

Sodium iodoacetate, sodium fluoride, sodium arsenate, 2–4 dinitrophenol (2,4-DNP) and monofluoroacetic acid (MFA) were obtained from Sigma. They were dissolved in medium and diluted to give appropriate concentrations which could be added in a volume of 0.1 ml to the culture medium. All solutions were sterilized by membrane filtration before use. Appropriate cell numbers which could be expected to yield 100 survivors were plated in 40 ml flasks (Nunc) containing 5 ml medium at least 6 h before addition of the analogues. The cells were irradiated 24 h after plating using a Cobalt 60 treatment unit delivering 2.0 Gy/min at 60 cms SSD. Three hours after irradiation all cultures received a medium change to remove the analogues. Survival was assessed using the colony formation assay of Puck and Marcus (1956). Cellular multiplicity was determined at the time of irradiation by counting the number of cells in a hundred randomly selected microcolonies.

The effect of the inhibitors on growth and glucose breakdown was assessed daily for seven days by counting the cells and estimating the amount of glucose used and lactate produced in the medium from cultures seeded initially with 5×10^4 cells. Glucose and lactate levels were measured in per-

chloric acid extracts of medium samples using the method of Schmidt (1961) for glucose and Gutmann and Wahlefeld (1974) for lactate.

Results

Effect of glycolysis inhibitors on the radiation response of CHO-K1 cells

Table 1 shows the results of an experiment designed to determine the dose effect relationship after 18 h exposure of the cells to a wide range of concentrations of sodium iodoacetate (NaIod), sodium fluoride (NaF) and sodium arsenate (NaAs) prior to receiving a single dose of 10 Gy gamma rays. It is apparent that a range of concentrations of each substance can be found which reduces the survival of CHO-K1 cells subsequently exposed to irradiation.

To investigate this further the length of time of exposure to the inhibitors was varied between 18 h pre-exposure and 0 h pre-exposure, in the latter case the substance was added to medium just prior to irradiation. In all cases the analogues were left in contact with the cells for 3 h after irradiation so as not to disturb the cells too soon. The results (Fig. 1 a–c) show that high concentrations of the inhibitors had a time-dependent effect while lower concentrations produced a less significant but constant effect irrespective of the length of time of exposure.

Effect of glycolysis inhibitors on the shape of the radiation survival curve

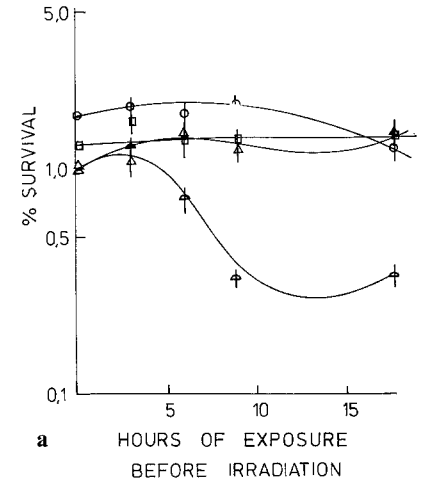
Figures 2 (NaF), 3 (NaIod) and 4 (NaAs) and Table 2 show the effect of the glycolysis inhibitors on the survival curve of CHO-K1 cells exposed for 18 h prior to irradiation.

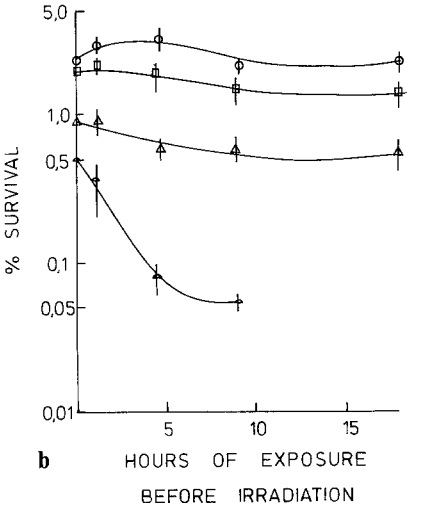
The concentrations of analogues chosen were the lowest which gave a consistent and statistically significant radiobiological response after 18 h exposure (see Table 1).

The results indicate that with all the inhibitors the survival of irradiated cells is severely reduced and the effect of each substance is to reduce the size of the survival curve shoulder. In the case of sodium iodoacetate and sodium fluoride, but not sodium arsenate, a small decrease in Do can be seen (Table 2).

Effect of glycolysis inhibitors on growth plating efficiency and multiplicity of CHO-K1 cells

The results presented so far clearly demonstrate a radiobiological effect of glycolysis inhibitors which is detectable after corrections have been made for variations in the unirradiated plating efficiency of control and treated cells. However, toxicity in general can be related to membrane or cellular damage and to alterations in cell cycle, all of which can effect the radiation response (Alper 1979). Therefore it was felt important to assess the contribution, if any, of toxicity to the observed results.





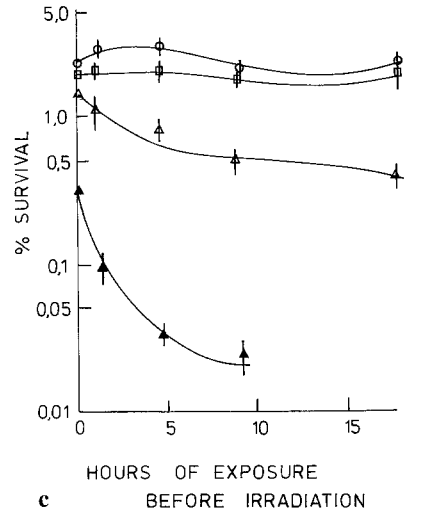


Fig. 1. a The effect on the % survival of CHO-K1 cells of increasing time of exposure to ordinary medium (○), 0.01 mM sodium fluoride (□), 1 m*M* sodium fluoride (△) or 5 m*M* sodium fluoride (□). (Experiments were done in triplicate and each point represents the mean of nine experimental points. Error bars are standard errors for $n=9$. Irradiation took place exactly 24 h after the cells were plated).
b The effect on the % survival of CHO-K1 cells of increasing time of exposure to 0.001 m*M* (□), 0.005 m*M* (△), 0.01 m*M* (□) of sodium iodoacetate or medium control (○) before irradiation to 10 Gy. (Experiments were done in triplicate and each point represents the mean of nine experimental points. Error bars are standard errors for $n=9$. Irradiation took place exactly 24 h after the cells were plated).
c The effect on the % survival of CHO-K1 cells of increasing time of exposure to 0.01 m*M* (□), 0.05 m*M* (△), 0.1 m*M* (▲) of sodium arsenate or medium control (○) before irradiation to 10 Gy. (Experiments were done in triplicate and each point represents the mean of nine experimental points. Error bars are standard errors for $n=9$. Irradiation took place exactly 24 h after the cells were plated)

Table 1. The effect of 18 h pre-exposure to increasing concentrations of glycolysis inhibitors on the relative survival of CHO-K1 cells irradiated to 10 Gy. Survival values have been adjusted so that the irradiated control survival = 100%. Errors are expressed as % standard error for $n=3$

Treatment	Relative survival
10 Gy only	100.0±9.2%[a]
10 Gy + 0.01 m*M* NaFluoride	82.4±6.3%
10 Gy + 0.1 m*M* NaFluoride	87.6±8.8%
10 Gy + 1.0 m*M* NaFluoride	85.2±7.4%
10 Gy + 5.0 m*M* NaFluoride	6.0±0.3%
10 Gy + 0.0001 m*M* NaIodoacetate	75.6±8.5%
10 Gy + 0.001 m*M* NaIodoacetate	48.4±6.3%
10 Gy + 0.005 m*M* NaIodoacetate	11.4±2.3%
10 Gy + 0.01 m*M* NaArsenate	58.0±6.2%
10 Gy + 0.05 m*M* NaArsenate	23.6±1.8%
10 Gy + 0.1 m*M* NaArsenate	8.8±0.6%

[a] Absolute survival after 10 Gy = 8.9±0.62

Table 2. The effect of 18 h pre-treatment with glycolysis inhibitors on the radiation survival curve parameters "n" and Do for CHO-K1 cells. Pooled results for 3 separate experiments. "n" values have been corrected for multiplicity

Treatment	"n y"	Do (Gy)
Control	6.3	1.4
5 m*M* NaFluoride	1.1	1.2
0.005 m*M* NaIodoacetate	3.0	1.2
0.05 m*M* NaArsenate	2.0	1.4

Table 3a. The plating efficiency and 24 h multiplicity of CHO-K1 cells exposed to increasing concentrations of glycolysis inhibitors for 18 h. (The inhibitors were added 6 h after plating the cells). Errors are the standard error of the mean for $n=9$

Treatment	% plating efficiency	24 h multiplicity
Control	86 ±7.4	3.2±0.3
0.01 m*M* NaFluoride	85 ±3.2	3.9±0.4
0.1 m*M* NaFluoride	86 ±4.3	3.3±0.3
1.0 m*M* NaFluoride	87 ±6.4	3.2±0.1
5.0 m*M* NaFluoride	45 ±4.8	2.2±0.4
0.0001 m*M* NaIodoacetate	84 ±5.8	3.7±0.4
0.001 m*M* NaIodoacetate	87 ±8.2	3.3±0.4
0.005 m*M* NaIodoacetate	83 ±7.2	3.3±0.3
0.01 m*M* NaIodoacetate	8.4±0.7	2.1±0.1
0.01 m*M* NaArsenate	84 ±6.2	3.4±0.5
0.5 m*M* NaArsenate	78 ±4.3	3.3±0.3
0.1 m*M* NaArsenate	18 ±0.9	2.4±0.3

Table 3a shows the effect of 18 h treatment with the inhibitors on 24 h plating efficiency and multiplicity. It is apparent that radiobiological effects can be observed at levels of inhibitors which are not in themselves toxic to the cells. This would argue against a significant involvement of cell cycle inhibition in the radiobiological effect. The data on growth, lactate and glucose breakdown (Table 3b) show that the levels of the inhibitors which

Table 3b. The effect of 48 h treatment with glycolysis inhibitors on the breakdown of glucose, accumulation of lactate and on the growth of CHO-K1 cells. (Errors are the standard error of the mean for $n=9$).
Initial concentration of glucose = 48.5 mM/5 ml
Initial concentration of lactate = 2.2 mM/5 ml
Initial cell number plated = $5.4 \pm 0.05 \times 10^4$

Treatment	Glucose	Lactate	Cell No ($\times 10^5$)
Control	38.3 ± 2.4	18.1 ± 0.63	5.4 ± 0.23
0.01 mM NaFluoride	40.9 ± 0.79	15.0 ± 0.62	7.33 ± 0.16
0.1 mM NaFluoride	40.8 ± 2.0	12.8 ± 0.84	6.92 ± 0.18
1.0 mM NaFluoride	40.0 ± 2.5	14.4 ± 0.1	5.25 ± 0.32
5.0 mM NaFluoride	44.3 ± 2.3	10.3 ± 0.3	3.2 ± 0.35
0.0001 mM NaIodoacetate	39.3 ± 0.4	15.0 ± 0.4	5.3 ± 0.22
0.001 mM NaIodoacetate	40.4 ± 1.8	11.0 ± 0.8	5.2 ± 0.19
0.005 mM NaIodoacetate	40.6 ± 2.2	8.0 ± 0.8	5.0 ± 0.3
0.01 mM NaIodoacetate	39.8 ± 0.7	3.4 ± 0.2	0.41 ± 0.03
0.01 mM NaArsenate	38.4 ± 2.4	18.4 ± 1.9	5.0 ± 0.3
0.05 mM NaArsenate	38.6 ± 3.3	17.6 ± 2.2	4.4 ± 0.5
0.1 mM NaArsenate	40.4 ± 4.2	17.4 ± 2.3	1.8 ± 0.09

cause radiobiological effects do block glycolysis, as shown by the reduced lactate production, except in the special case of NaAs where no inhibition of glycolysis is observed. The lack of effect of the inhibitors on the initial glucose levels indicates that the cells' ability to perform the initial glycolytic reactions is unaffected by the inhibitors which only block specific glycolytic enzymes.

The effect of respiratory inhibitors on the radiation response

Table 4 shows that neither 2,4-DMP nor MFA over a wide range of concentrations had any effect on the growth or radiation response of CHO-K1 cells.

Discussion

A common feature of all the inhibitors tested is that they reduced the survival of CHO-K1 cells exposed subsequently to irradiation. The constancy of the effect of low levels of the inhibitors with time of exposure prior to irradiation argues against a significant involvement of toxic effects in the radiobiological response, although for the high levels of each inhibitor toxic effects are obvious. It is interesting that the threshold concentration for toxicity (cell death in the absence of radiation) is higher than the threshold for a combination of radiation and inhibitor. At all levels of inhibitor tested a synergistic effect of the two factors is apparent since the radiation survival data have all been corrected for any reduction in P.E. due to toxicity. The results are similar to those obtained in this laboratory using glu-

Table 4. The effect of the respiratory inhibitors 2,4-dinitrophenol (2,4-DNP) and monofluoroacetic acid (MFA) on the survival of irradiated (10 Gy) and unirradiated CHO-K1 cells. The survival for irradiated cells has been corrected for variation in the relevant control plating efficiency

Treatment	Unirradiated	Irradiated	Significance[a]
Control	89.3± 8.4	4.3±0.4	
0.01 m*M* 2,4-DNP	93.1± 2.7	4.7±0.3	NS
0.1 m*M* 2,4-DNP	92.9±11.2	4.5±0.1	NS
1.0 m*M* 2,4-DNP	84.4± 9.2	4.2±0.3	NS
0.0001 m*M* MFA	88.5± 6.3	4.8±0.4	NS
0.001 m*M* MFA	89.2± 3.4	4.4±0.2	NS
0.01 m*M* MFA	84.0± 5.8	6.3±0.8	NS
0.1 m*M* MFA	90.7± 6.8	5.1±0.3	NS
1.0 m*M* NFA	84.8± 7.2	5.5±0.3	NS
10.0 m*M* MFA	77.6± 8.8	3.1±0.6	NS

[a] T-test for $n=3$

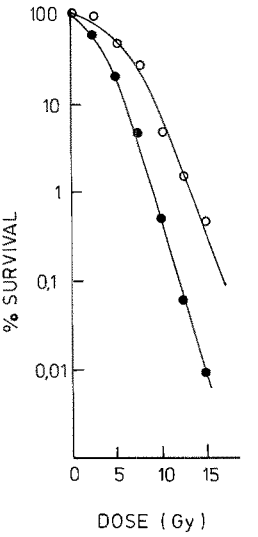


Fig. 2. Primary curves for control CHO-K1 cells (o) and those treated with 0.005 m*M* sodium iodoacetate (•) for 18 h prior to irradiation. Standard errors were contained within the experimental points

cose analogues and lactate under the same conditions and strongly suggest that inhibition of glycolytic energy metabolism by a variety of methods results in a common radiobiological response in CHO-K1 cells. The main consequences for cultured cells of inhibition of glycolytic metabolism are likely to be a reduction in cellular ATP levels or loss of metabolic precursors (Lehninger 1983). The occurrence of the response after treatment of cells with sodium arsenate could suggest that inhibition of ATP production is the key factor since arsenate allows glycolysis to proceed while preventing substrate level phosphorylation, thus depriving the cell of any net gain of ATP but maintaining levels of glycolytic metabolites (Lehninger 1983).

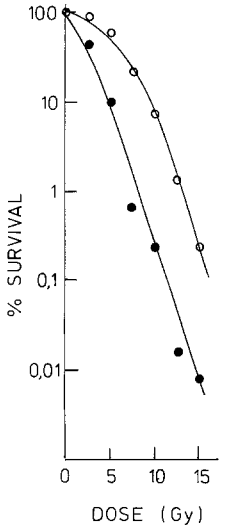


Fig. 3. Primary survival curves for control CHO-K1 cells (o) or those treated with 5 m*M* sodium fluoride (•) are 18 h prior to irradiation. Standard errors ($n=6$) are contained within the experimental points

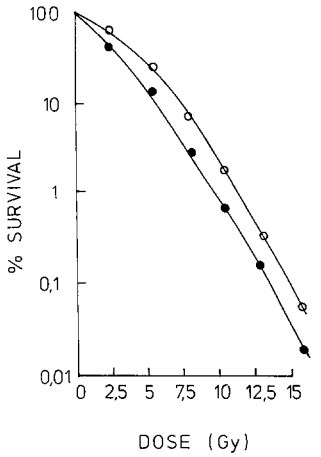


Fig. 4. Primary survival curves for control CHO-K1 cells (o) and those treated with 0.05 m*M* sodium arsenate (•) for 18 h prior to irradiation. Standard errors were contained within the experimental points

A central role for ATP in the mechanism is also suggested by the results obtained when the two respiratory inhibitors (MFA and 2,4-DNP) were used; these substances had no effect on the radiobiological response of the cells over a wide range of concentrations. They also were completely nontoxic and did not affect the levels of lactate formed over a seven day growth period (Seymour 1983). This can be taken as reasonable evidence that CHO-K1 cells mainly respire anaerobically under our laboratory conditions and presumably, therefore, derive their ATP from anaerobic glycolysis. Inhibition of this process results in a reduction in the cells' ability to survive a dose of radiation, while the use of a respiratory inhibitor (2,4-DNP) which in other cells reduces both ATP and radiation survival (Nishizawa et al. 1979) had no effect on our cells.

A role for ATP in the response of cells to radiation has been suggested by Kiefer (1971) who concluded that an essential requirement for repair of radiation damage in yeast was energy rich metabolites, for example ATP.

Bryant (1976) also concluded that Elkind recovery was an energy-dependent process and Alper (1979) suggested that the radiation response of aerobic cells could be influenced by the rate of respiration permitted by the available oxygen. A similar result was obtained by Ashby et al. (1969) using He-La cells, who showed that when glycolysis was blocked using sodium fluoride, the cells bypassed the enolase reaction and were able to maintain their previous level of ATP production and overcome the inhibitor induced reduction in radiation survival.

In summary, if CHO-K1 cells are exposed to inhibitors of glycolysis their survival following irradiation is reduced. The effect is likely to be related to depletion of ATP levels and would support suggestions by other authors that cellular ATP is a critical factor in the prevention and/or repair of radiation damage.

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