Effects of X-irradiation on the Oxygen Consumption and Lysine Uptake of HeLa Cells in the Presence of Metabolic Substrates and Inhibitors

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培養 HeLa 細胞의 酸素消費量과 Lysine 吸收에 미치는 X-線 照射의 影響

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摘 要

培養 HeLa 細胞의 酸素消費量과 lysine 吸收量에 미치는 X-線 照射의 영향을 측정하였다.

1. 200 r 의 X-線 照射는 酸素消費量에 아무러한 영향이 없다. 酸素消費量은 succinate, citrate, 및 α-ketoglutarate 에 의하여 增大되며 X-線 照射群에서도 이 傾向은 同一하다.

2. 酸素消費量에 미치는 sodium azide 와 2, 4-dinitrophenol 의 영향은 X-線 照射에 의하여 상당히 변화된다.

3. Lysine 의 初期吸收率은 X-線 照射에 의하여 甚히 低下되며, 또한 飽和吸收量도 減少된다.

4. Glucose 는 lysine 의 吸收를 促進시키고, succinate 는 아무러한 영향이 없으며 citrate 와 α-ketoglutarate 는 억제한다. X-線 照射는 이러한 傾向에 아무런 변화를 초래하지 않는다.

5. Lysine 의 吸收에 미치는 sodium azide 와 2, 4-dinitrophenol 의 영향은 酸素消費量에 미치는 이들의 영향과 判異하나. 이러한 傾向은 X-線 照射群에서도 대체로 동일하다.

INTRODUCTION

Studies on the metabolism of cancer cells have been extensively carried out because cancer is considered as the result of a defect of the homeostatic mechanisms which maintain the balance among cells in an organism. Since the nature of these mechanisms is not understood, nothing is known of the lesion which causes the disease. It is clear that the primary lesion occurs in the cell. The tissue culture method therefore has been employed to study the cell lesion in isolation from the host.

Since cancer cells have been in general considered physiologically that, as in their chromosomal morphology, their metabolic activities should be somewhat different from those of normal cells, differences in metabolic patterns, enzyme systems, metabolic responses to various substances, and the pattern of amino acid accumulation between cancer and nonmalignant cells have been widely studied by many workers.

Shonk *et al.* (1964) studied the patterns of the glycolytic enzymes in several different human tissues in order to distinguish the patterns of malignant tissues from those of normal ones. Banerjee *et al.* (1965) reported that some of the TCA cycle enzymes show higher, while others lower, activities in malignant than in normal cells of human cervical tissues. Bickis and Quastel (1965) reported the effects of several well-known metabolic inhibitors on energy yielding processes and on operations such as protein and nucleic acid biosynthesis in Ehrlich ascites carcinoma cells. Bickis and Henderson (1966)

have measured the respiration, glycolysis and radioactive tracer incorporation into protein and nucleic acids and have established the metabolic malignancy index from the sum of the three metabolic parameters. Mahaley(1966) measured the respiration of normal brain and brain tumors and reported that the degree of malignancy of brain tumors was inversely proportional to the respiratory rate. McKee *et al.* (1966) reported that there are striking differences among the Krebs cycle intermediates and lactate and pyruvate as to their ability to stimulate respiration of washed Ehrlich-Lettre carcinoma cells in an endogenous system and to allow an observable Crabtree effect when glucose is added.

Studies on the amino acid uptake, on the other hand, by malignant cells have been also carried out extensively because tumor cells show characteristic selectivities in the uptake of specific amino acids. O'Neal and Griffin(1963) reported that approximately 30–70 $\mu\mu$moles of amino acid/mg ribosomal protein were incorporated into Novikoff ascites tumor cells when C^{14}–valine, phenylalanine, or lysine was added to the medium, and that the addition of RNA preparations from tumor caused a 16–45% increase in amino acid incorporation. Inhibition of amino acid incorporation into protein of Yoshida ascites hepatoma cells by glyceraldehyde was reported by Guidotti *et al.* (1964). Inhibition of amino acid transport by metabolic inhibitors in Ehrlich ascites cells was also reported by Jacquez and Sherman (1965). Stimulation of aromatic amino acid transport by *p*-fluorophenylalanine in the sarcoma 37 cell was reported by Guroff *et al.* (1964). Banerjee *et al.* (1965) reported that some amino acids were oxidized at a higher rate in malignant than in normal cells. Bickis and Quastel (1965), studying the incroporation of C^{14}-glycine into proteins of Ehrlich ascites carcinoma cells in the presence or absence of various metabolic inhibitors, reported that it was possible to estimate approximately the amount of uncoupling of phosphorylation from oxidation that occurs with inhibitors which act solely as uncouplers and thereby that amount of tumor respiration which is coupled to biosynthetic processes.

The effect of x-irradiation on the metabolism of cancer cells is, however, studied poorly although the irradiation is widely employed for the therapy of the cancer. Killander *et al.* (1962) studied the effect of x-irradiation on nucleotide content and reported that the irradiation caused an increase in the DNA and other UV-absorbing compounds in the nuclei. It is therefore desirable to study the effect of ionizing radiation on the metabolism of cancer cells.

Present study, as a part of research project entitled "Studies on radiosensitivity of cultured human normal and cancer cells", was carried out to determine the effects of x-irradiation on the utilization of carbohydrate metabolites, on the response of the cell metabolism to the metabolic inhibitors, and on the uptake of lysine in the presence or absence of carbohydrate metabolites and metabolic inhibitors using cultured HeLa cells.

MATERIALS AND METHODS

HeLa cells cultured in TC Medium 199 (Difco Laboratories, Detroit, Michigan, USA) in this laboratory were suspended in Ca-free Krebs-Ringer phosphate buffer of pH 7.4 (Umbreit *et al.*, 1964) after the irradiation to 200 r of x-rays. X-irradiation was made with an x-ray generator of General Electric Maxitron 250–III at 230 kv and 10 ma with TH_2 filter(0.4 mm Sn + 0.25 mm Cu + 1.0 mm Al) at the dose rate of 19 r/min. Dosage was measured with a Victoreen γ-meter. After preparing Warburg vessels with Ca-free Krebs-Ringer phosphate buffer solution in the main portion containing glucose, succinate, citrate or α-ketoglutarate in the concentrations that would give a final concentration of 10mM each after the addition of the cell suspension, and a filter paper strip soaked with 0.2 ml of 20% KOH in the center well, the cell suspension was added just prior to placing the manometers in the water bath. ATP was added in the incubation mixture in the final concentration of 2 mM. Metabolic inhibitors, sodium azide (NaN_3) and 2,4-dinitrophenol (DNP), in the final concentrations of 5 mM and 0.2 mM respectively were put in the sidearms of the manometer vessels. These concentrations were employed after preliminary experiments. With air in the gas phase and the bath temperature at 37°C, the flasks were equilibrated for 30 minutes, the taps were closed, and incubation continued for 120 minutes, during which time the manometers were read at 15 minute intervals by the direct reading method (Umbreit *et al.*, 1964). Total volume of the incubation mixture was 2 ml. After the

incubation, the content of the vessels was homogenized and analyzed for nitrogen by micro-Kjeldahl and electrophotometric methods. One ml of the cell suspension gave an average of 0.52 mg of nitrogen.

Lysine uptake was measured by incubation of the irradiated cells in a test tube containing Krebs-Ringer phosphate solution with glucose, succinate, citrate or α-ketoglutarate in the final concentrations of 10 mM each and 2 mM ATP, in the presence or absence of 5 mM NaN_3 or 0.2 mM DNP. Incubation was done with a metabolic shaker at 37°C with a constant shaking rate. Total volume of the incubation medium was 2 ml.

The tracer amino acid, L-lysine-U-C^{14} in the specific activity of 230 mCi/mM, purchased from Volk Radiochemical Co., Chicago, USA, was dissolved with carrier amino acid in the final concentration of 2 mM in Krebs-Ringer phosphate buffer. After 20 minutes of preincubation, the tracer was added to the incubation medium and the incubation continued for 30 minutes. At the end of the incubation, tubes were taken out and centrifuged immediately at high speed for one minute to separate the cells from the incubation medium. The supernatant was discarded and the cell sediment was quickly washed with ice-cold Krebs-Ringer phosphate buffer by rapid centrifugation and the washing was repeated twice. Cells thus washed were resuspended in distilled water, allowed to lyse for 1 hour, and cell debris were removed by centrifugation. Radioactivity was counted on the supernatant with a windowless, gas-flow type GM counter. Counting the radioactivity on the cell debris revealed no appreciable contamination or adsorption of the tracer on the fragments. These cell debris were used for the determination of the nitrogen content which was done by the micro-Kjeldahl and by the electrophotometric methods. One ml of cell suspension was equivalent to 0.45-0.52 mg nitrogen.

RESULTS AND DISCUSSION

The oxygen consumption of control and x-irradiated HeLa cells in the medium containing glucose, succinate, citrate, or α-ketoglutarate, with or without the metabolic inhibitor, NaN_3 or DNP, is shown in Table 1. In Table 2 are shown the relative values of the QO_2 calculated from figures in Table 1, the value obtained in media in the control group being taken as 100.0, for the convenience of evaluating the effects of x-irradiation on the oxygen consumption.

As shown in Tables 1 and 2, it is hard to see any singnificant difference in the oxygen consumption of irradiated HeLa cells from that of control cells (not irradiated HeLa cells); i. e., in the glucose medium, irradiated HeLa cells show 95.3% QO_2 of control cells, the difference being only about 5 % which may be completely negligible.

Table 1. Oxygen consumption of x-irradiated HeLa cells in various medium compositions.

Treatment	Inhibitor	Substrate			
		Glucose	Succinate	Citrate	α-Ketoglutarate
Control	- -	0.427*	0.768	0.546	0.599
	NaN_3	0.196	0.248	0.139	0.345
	DNP	0.195	0.554	0.304	0.247
Irradiated	- -	0.407	0.773	0.608	0.662
	NaN_3	0.175	0.446	0.108	- -
	DNP	0.246	0.714	0.200	0.800

* Figures are μlO_2/hr/ml suspension and are average of three to five determinations.

Table 2. The effect of x-irradiation on the oxygen consumption of HeLa cells in various medium compositions.

Treatment	Substrate			
	Glucose	Succinate	Citrate	α-Ketoglutarate
Control	100.0	100.0	100.0	100.0
Irradiated	95.3	101.7	111.1	110.3

Similar phenomena are seen in the medium containing succinate, citrate or α-ketoglutarate in place of glucose. In the citrate and α-ketoglutarate media, about 10% increment in the oxygen consumption of irradiated group was seen. Statistical treatment, however, showed that this amount of difference may be hardly regarded as to mean significant. Figures in the above tables are those obtained at one or two hours after the irradiation.

Measurements at 24 hours after the irradiation gave essentially the same result as those presented in the tables. X-irradiation of 200 r thus seems to have no immediate effect on the aerobic respiration of the cell while a significant morphological change in the chromosomes of cancer cells irradiated with 60 r of x-rays has been reported to occur.

The amounts of oxygen consumed by HeLa cells were different according to the type of substrates added as seen in Table 3.

Table 3. The relative effects of added substrates on the oxygen consumption of x-irradiated HeLa cells.

Treatment	Substrate			
	Glucose	Succinate	Citrate	α-Ketoglutarate
Control	100	180	128	140
Irradiated	100	190	150	163

When the QO_2 obtained in the medium containing glucose was taken as 100, the relative values of QO_2 in the medium containing succinate, citrate, or α-ketoglutarate were 180, 128, and 140 respectively in the control group, and 190, 150 and 163 in the irradiated group. Among substrates added in place of glucose, succinate was by far the most effective in increasing the oxygen consumption, the values in both groups approaching to nearly twice that of glucose medium. Though less effective than succinate, citrate and α-ketoglutarate also increased the oxygen consumption of both control and irradiated cells. Thus, the increase in the oxygen consumption by the added substances is seen in both control and experimental groups and the degree of increment was somewhat different from one substrate to another. Furthermore, the degree of increment in the oxygen consumption by the added substrates seems to be slightly higher in the irradiated group than in the control. Whether this difference in the effectiveness of the added substrate is due to the irradiation or to the experimental error is hard to indicate presently.

McKee *et al.* (1966) reported that there were striking differences among Krebs cycle intermediates as to their ability to stimulate respiration of Ehrlich-Lettre carcinoma cells and that succinate was very effective in producing respiratory stimulation while citrate and α-ketoglutarate were not. The results obtained in the present experiment are generally in good agreement with that of McKee, though citrate and α-ketoglutarate in the present study showed somewhat greater stimulation than McKee reported.

The effects of metabolic inhibitors on the oxygen consumption of irradiated HeLa cells were measured using 0.2 mM DNP and 5 mM NaN_3 in the medium containing

Table 4. Relative effects of NaN_3 and DNP on the oxygen consumption of HeLa cells.

Treatment	Inhibitor	Substrate			
		Glucose	Succinate	Citrate	α-Ketoglutarate
	--	100.0	100.0	100.0	100.0
Control	NaN_3	45.9	32.3	25.5	57.6
	DNP	45.6	72.1	55.7	41.2
	--	100.0	100.0	100.0	100.0
Irradiated	NaN_3	42.9	57.7	17.7	?
	DNP	58.9	92.4	32.9	120.8

glucose, succinate, citrate or α-ketoglutarate and the results are summarized in Table 4 with relative values. The concentrations of the two inhibitors employed were such that about half oxygen consumption in the glucose medium be given when they are added to the medium.

Table 4 shows that NaN_3 and DNP, in the concentrations that reduce the oxygen consumption of control HeLa cells in the glucose medium by half, act differently according to the different substrate added in the medium. In the control group, the oxygen consumption of cells in succinate medium was more inhibited by NaN_3 and less by DNP than it was in glucose medium. A similar effect of NaN_3 and DNP was also seen in the citrate medium. Effects of NaN_3 and DNP in α-ketoglutarate medium appeared more or less in the opposite direction. The inhibitory effects of NaN_3 and DNP in the irradiated group were reduced in the succinate medium especially in the case of DNP. In the citrate medium, on the other hand, the inhibitory effects of the inhibitors were enhanced and the relative values of the oxygen consumption in this group are less than in the control group. The presence of DNP along with α-ketoglutarate in the medium apparently increases the oxygen consumption of the irradiated cells in contrast to its inhibitory effect in the control cells. Measurement of the effect of NaN_3 in the α-ketoglutarate-containing medium of the irradiated group was failed.

Mahaley (1966) measured the respiration of normal brain and brain tumors to evaluate the effects of various anticancer drugs upon respiration and reported that the respiration of normal brain tissue was inhibited by each anticancer drug used, whereas the response of brain tumors was variable. The respiratory response of HeLa cells to the metabolic inhibitors seems to be altered by the x-irradiation, the details of which mechanism are not known presently.

The time course of the uptake of lysine-C^{14} by HeLa cells, both control and x-irradiated, in the medium containing 10 mM glucose and 2 mM ATP is shown in Fig. 1. Both groups attained an uptake saturation at about 20 minutes of incubation. The amount of lysine accumulated in the cells at saturation was greater in control group than in irradiated cells. The initial rate of uptake

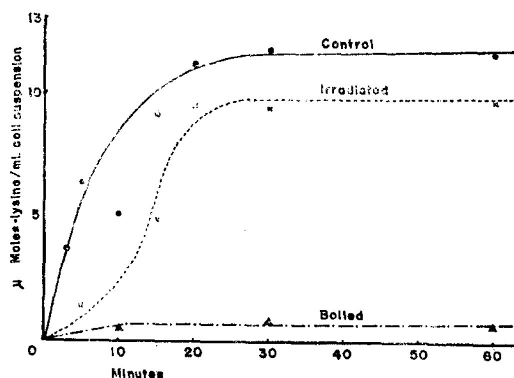

Fig. 1. Time course of lysine uptake of HeLa cells. Each point represents the average of duplicate incubations. Incubation medium consists of Ca-free Krebs-Ringer phosphate buffer with 10 mM glucose plus 2 mM ATP.

was higher in control cells than in irradiated cells; the amounts of lysine taken up by control cells during the first 15 minutes of incubation were nearly twice the amount by irradiated cells. To check the adsorption of the isotope on the cell surface, an equivalent amount of cells which had been previously boiled for 10 minutes was incubated simultaneously and treated the same way as living cells to measure the radioactivity. The amount of adsorbed or contaminated radioactivity was negligible comparing with that of the accumulated activity as shown in Fig. 1. The irradiation of HeLa cells to x-rays thus decreases the initial uptake rate of lysine, although the saturation comes at about the same time with that of control cells.

The uptake of lysine-C^{14} by the irradiated HeLa cells in the medium containing glucose, succinate, citrate or α-ketoglutarate with the presence or absence of NaN_3 or DNP was measured and the results are shown in Table 5.

For the convenience of evaluating the effect of x-irradiation on the lysine uptake, relative values of the lysine uptake calculated from Table 5 are given in Table 6, where the values of control group in each medium are taken as 100.0.

In order to compare the effects of substrate in the control with those in the irradiated group, relative values of the lysine uptake in various medium compositions are shown in Table 7, the value obtained in the medium

Table 5. The uptake of lysine by the x-irradiated HeLa cells in various medium compositions.

Treatment	Inhibitor	Substrate				
		None	Glucose	Succinate	Citrate	α-Ketoglutarate
	--	9.67*	13.93	10.65	7.94	7.08
Control	NaN_3	--	12.50	5.20	3.30	3.30
	DNP	--	12.10	5.17	3.75	3.98
	--	8.10	11.16	8.72	4.48	6.03
Irradiated	NaN_3	--	10.17	3.83	2.70	3.18
	DNP	--	9.45	4.36	3.63	3.60

* Figures are μmoles lysine per ml cell supension and are average values of triplicate incubations.

Table 6. The effect of x-irradiation on the uptake of lysine in various medium compositions as expressed in relative values.

Treatment	Inhibitor	Substrate				
		None	Glucose	Succinate	Citrate	α-Ketoglutarate
	--	100.0	100.0	100.0	100.0	100.0
Control	NaN_3	--	89.6	48.8	41.5	46.6
	DNP	--	86.8	48.5	47.2	56.3
	--	83.7	79.8	81.9	56.4	85.2
Irradiated	NaN_3	--	72.8	36.8	34.0	45.0
	DNP	--	67.7	40.8	45.7	50.8

Table 7. The effects of metabolic substrates on the uptake of lysine in various medium compositions as expressed in relative values.

Treatment	Inhibitor	Substrate				
		None	Glucose	Succinate	Citrate	α-Ketoglutarate
	--	100.0	144.3	110.2	82.1	73.1
Control	NaN_3	--	129.1	53.6	34.1	34.1
	DNP	--	125.0	53.4	38.7	41.1
	--	100.0	137.5	107.8	55.3	74.5
Irradiated	NaN_3	--	125.3	42.3	33.3	39.2
	DNP	--	116.5	53.8	44.8	44.4

which contained no metabolic substrate being taken as 100.0. The effects of metabolic inhibitors in the uptake of lysine as expressed in values relative to the inhibitor-free system are given in Table 8.

As shown in the above four tables, it is quite apparent that x-irradiation inhibits the uptake of lysine significantly; the amount of lysine accumulated in the irradiated cells was less than that of control cells in every experimental condition tested. The effect of irradiation thus seems to cause alterations in the characteristic transport of amino acid as well as alterations in morphological aspect. According to Burke (1962), rat liver undergoing carcinogenesis or regeneration has decreased catabolism of amino acids and increased synthesis of protein. The decrease in the lysine uptake by the irradiation then may be thought to be caused by the damage in protein syn-

thetic systems in the cell. Whether the decrease in the lysine uptake by the irradiation is due to the alterations in the transport system of the cell membrane or to the decreased protein synthesis in the cell is not known but, since the initial rate of the uptake is much decreased by the irradiation (Fig. 1), it seems probable that the transport system is altered by the irradiation.

The presence of glucose in the medium increases the lysine uptake in both control and irradiated Hela cells. This increment is a little higher in control than in irradiated cells (Table 7). Even in the presence of NaN_3 or DNP in the same concentrations with those used in the determination of oxygen consumption, the amounts of lysine taken up by the cells of both groups were greater than those in Krebs-Ringer medium.

Table 8. The effects of metabolic inhibitors on the uptake of lysine in various medium compositions as expressed in relative values.

Treatment	Inhibitor	Substrate				
		None	Glucose	Succinate	Citrate	α-Ketoglutarate
	– –	100.0	100.0	100.0	100.0	100.0
Control	NaN_3	– –	89.6	48.8	41.5	46.6
	DNP	– –	86.8	48.5	47.2	56.3
	– –	100.0	100.0	100.0	100.0	100.0
Irradiated	NaN_3	– –	91.1	66.8	60.3	52.8
	DNP	– –	84.7	76.2	81.1	59.8

Contrast to the enhancing effect of glucose, the presence of succinate has no significant stimulating effect on the lysine uptake. The effects of citrate and α-ketoglutarate on the lysine uptake appeared to be also inhibitive in both control and test groups. According to Annegess (1963), increased concentration of glucose in the lumen reduces the absorption rates of glycine, methionine, histidine and glutamic acid, and galactose reduces alanine, methionine, lysine, histidine and glutamic acid absorption in the dog. He reported also that fructose did not reduce the absorption of the above amino acids. Although the results reported by Annegess were obtained from dogs perfused with amino acids and hexoses and therefore may not be directly applicable to the present study, it may be concluded that the amino acid absorption is greatly affected by the metabolites in the medium. It is interesting to note that succinate which greatly increases the oxygen consumption of the cell does not increase the lysine uptake, and that citrate and α-ketoglutarate, both of which considerably enhance the respiratory rate of the cell, do not augment but conversely inhibit the lysine uptake of the same cells.

The effects of NaN_3 and DNP on the uptake of lysine are generally very inhibitive in both control and test cells (Table 8). The inhibitors have, however, practically no effect on the lysine uptake when added to the glucose medium where they cause about 50% inhibition in oxygen consumption. The inhibitory effects of NaN_3 and DNP on the lysine uptake, therefore, seem to be different from those seen in the oxygen consumption. X-irradiation of the cell seems to diminish the inhibitory effect of the inhibitors. However, since x-irradiation decreases markedly the absorption of lysine as already described, the effects of the inhibitors on the lysine uptake in the irradiated cells may appear less apparently than in control cells.

SUMMARY

The effects of x-irradiation on the utilization of glucose, succinate, citrate and α-ketoglutarate, on the response of the cell metabolism to NaN_3 and DNP, and on the uptake of lysine in the presence or absence of the metabolites or the inhibitors were studied using HeLa cells and the results are summarized as follows:

1. 200 r of x-irradiation had no immediate effect on the oxygen consumption of cells.

2. The oxygen consumption was greatly stimulated by succinate, α-ketoglutarate and citrate and in decreasing order and x-irradiation caused no remarkable change in this order.

3. The respiratory response of the cell to the metabolic inhibitors seems to be altered by x-irradiation.

4. The initial rate of the uptake of lysine was markedly retarded and the accumulation of lysine in the cell was decreased by irradiation.

5. Glucose increased the lysine uptake whereas succinate had no effect and citrate and α-ketoglutarate reduced the absorption. X-irradiation did not alter this tendency.

6. The inhibitory effects of NaN_3 and DNP on the lysine uptake were quite different from those seen in the oxygen consumption.

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